AD_____

Award Number: DAMD17-98-1-8144

TITLE: Mechanisms of Bone Metastasis from Breast Cancer Using a Clinically Relevant Model

PRINCIPAL INVESTIGATOR: Robin L. Anderson, Ph.D.

CONTRACTING ORGANIZATION: Peter MacCallum Cancer Institute
Victoria, Australia 3000

REPORT DATE: July 2001

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	July 2001	Final (1 Jul 98 - 30 Jun 01)

4. TITLE AND SUBTITLE
Mechanisms of Bone Metastasis from Breast Cancer Using a Clinically Relevant Model

5. FUNDING NUMBERS
DAMD17-98-1-8144

6. AUTHOR(S)
Robin L. Anderson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Peter MacCallum Cancer Institute
Victoria, Australia 3000

E-Mail: r.anderson@pmci.unimelb.edu.au

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
Report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Bone metastases develop in 70% of breast cancer patients who are left untreated or for whom treatment fails. Once the tumor has metastasised, the disease is hard to control and treatment options are limited. A lack of knowledge of the metastasis process and the genes that control it precludes the use of rational drug design. Breast cancer metastasis to bone is poorly understood largely due to a dearth of model systems available to study this process. We have developed a murine model of breast cancer that actively mimics the human disease. After implantation of tumor cells into the mammary gland, a primary tumour develops and subsequently metastasises to the lymph nodes, lung and bone. The resulting bone lysis, paralysis, hypercalcaemia and elevated plasma parathyroid hormone-related protein (PTHrP) are all hallmarks of the human disease.

We have used the 3 years of DOD funding to develop and characterise the model and to seek the genes that regulate metastasis to bone. By differential gene expression analysis using two clones that are both metastatic but differ in their ability to metastasise to bone, we have identified candidate genes for bone tumor homing and growth. We have also determined the expression patterns and function of genes already proposed by others to play a role in breast cancer metastasis.

14. SUBJECT TERMS
Breast cancer, murine model, metastases, bone, regulatory factors.

15. NUMBER OF PAGES
62

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

INTRODUCTION

Research into mechanisms of breast cancer metastasis to bone has been hampered by the lack of animal models that mimic the pattern of spread in humans. Most of the current models use immunocompromised mice and abnormal routes of introduction of the tumor cells to enable the tumor cells to reach bone. In addition, until now, there has been no model that allows a comparison of primary tumors and secondary metastases in bone with the same primary tumor metastasising to another site. The aim of this project is to determine the factors that are important in the development of breast cancer metastases in bone. To achieve this aim, we have developed the first murine model of spontaneous metastasis of breast cancer cells to bone. During the first year of this grant, we completed a detailed characterisation of this model. The purpose of this was to enable us to use selected clones with defined metastatic patterns to seek genes that control metastasis to bone. We are taking two approaches to achieve this aim. The first approach is to analyse in our improved model, the function of genes already implicated from other studies in bone metastasis. The other approach is to complete a genome wide screen for differentially expressed genes between clones that can or cannot metastasize to bone. Identification of such factors will facilitate the identification of specific therapies that target these factors and thereby reduce the incidence and morbidity associated with bone metastases.

BODY

The proposed specific aims of this project are as follows:

Task 1. To complete the characterization of the tumor model.

Task 2. To determine the association between the early detection of tumor cells in bone marrow with the subsequent development of overt metastases.

Task 3. To identify the factors that are required for metastasis to bone:

- by analysis of candidate genes.
- by genome wide screening for novel genes required for metastasis to bone.

Task 1: Characterisation of the tumor model

This was completed in the first year of the grant and presented in the 1999 annual report. The results have been published in a paper in Clinical & Experimental Metastasis in 1999 (copy of published paper attached). Integral to the model is the existence of multiple clones derived from the one spontaneous tumor. These clones have different metastatic properties, ranging from non-metastatic to weakly metastatic to aggressively metastatic, and vary in the sites of metastasis. These clones are described in the attached paper. Measurement of metastatic growth was achieved by conventional methods: lung nodule counts, clonal growth of drug resistant tumor cells recovered from a tissue and histology. However, our proposed experiments required a more accurate measure of tumor burden. Our initial plan was to tag the tumor cells by transfecting them with the gene encoding green fluorescent protein (GFP) and to then measure metastatic tumor burden by fluorescence emission. However, we found that expression of high levels of GFP by the tumor cells resulted in slower tumor growth and reduced metastasis, possibly due to immunological rejection of tumor cells presenting GFP peptides on class 1 MHC. We did manage to identify a tumor line expressing very low levels of GFP that metastasized with kinetics indistinguishable from the parental line, however GFP expression in this line was insufficient to allow its use as a tumor cell marker. Instead, we developed a semi-quantitative PCR assay based on the ratio of GFP DNA to vimentin DNA, that allowed us to detect transfected tumor cells not expressing detectable GFP. Subsequently, we developed a truly quantitative method of measuring tumor cell burden using a real time quantitative PCR (RTQ-PCR) assay based on the Taqman chemistry. This technique, based on the presence of the *neo* gene in the transfected tumor cells, can reliably and quantitatively detect small numbers of tumor cells in an organ. This assay is also useful for gain or loss of function experiments where the tumor cells are transduced with expression constructs for the gene of interest. These constructs also contain the gene for neomycin resistance *(neo)*, which is used to select successfully transduced cells. For analysis of metastatic tumor burden, tissue samples, including bone, are snap frozen in liquid nitrogen and pulverised at this temperature in a steel homogeniser. Aliquots of the powder are weighed and genomic DNA extracted. A multiplexed RTQ-PCR reaction measures the level of a selectable marker or reporter gene (GFP or *neo*) and an endogenous gene, vimentin, in the same sample. Comparison of the abundance of the tumor cell marker with that of the endogenous gene allows calculation of tumor cell burden. Although this method does not allow precise cell number measurement, it does enable tumor burden to be measured relative to a control group. This method, largely using *neo* as the reporter gene, is used extensively in the results given below.

To determine the sensitivity of the RTQ-PCR technique, we spiked a homogenate of bone from a non-tumor bearing mouse with tumor cells and established that we can detect approximately 100

tumor cells in 20 μg DNA which contains about 3 x 10^6 cells.

An analysis of tumor burden in multiple organs after implantation of the tumor cells into the mammary gland is underway for 66cl4, the line that metastasizes to lymph nodes and lung, and has been completed for 4T1.13, the line that metastasizes to lymph nodes, lung and bone. These lines have been tagged with *neo* as a reporter gene to facilitate real time PCR analysis. The results for 4T1.13 are presented in Figure 1. The greatest tumor burden is found in the femur and brain. Metastasis to brain will be a topic for future studies.

Task 2. Determination of the association between the early detection of tumor cells in bone marrow with the subsequent development of overt metastases.

The kinetics of metastatic spread of three sublines, 67NR, 66cl4 and 4T1, and the clonal line 4T1.2 derived from the original spontaneous carcinoma, were assessed in female BALB/c mice of 6-8 weeks of age following varying routes of inoculation. Micrometastases were first detected in the lung on day 15 after inoculation of 4T1 cells into the mammary fat pad. By day 22, micrometastases were first detected in the vertebrae but only with 4T1 cells. Tumor cells were never recovered from the vertebrae of mice inoculated with 67NR or 66cl4. Thus our data indicate that the non-bone metastasising lines also fail to home to the bone environment in numbers detectable in our assay. However, these early assays described in the 1999 publication, involved clonal growth of drug resistant tumor cells recovered from a tissue. When repeated using RTQ-PCR of the *neo* tagged cells, tumor cells were detected in the spines of mice inoculated with 66cl4, albeit much less than when the 4T1.2 or 4T1.13 clones were used. However, overt tumor deposits have not been detected in the spines of mice injected with 66cl4 suggesting that, in the time frame of this experiment, the presence of 66cl4 tumor cells in bone marrow did not predict the presence of tumor deposits.

A time course for metastatic spread using the bone metastasising clone, 4T1.2, is shown in Figure 2. Tumor cells were evident in the lung by day 14 and increased about 10-fold by day 28 (Figure 2A). Kinetics of spread to spine were slower, with tumor signal first obvious by day 28.(Figure 2B). Plasma calcium levels did not change during this 5 week period, being around 2.4 mM.

After intravenous injection, clonogenic 4T1 cells were recovered from lungs at all times. Lung metastases become grossly visible by day 19 and vertebrae became uniformly positive by day 17 following injection. An attempt to isolate a clone of 4T1 for which clonogenic cells were not detected in the vertebrae was unsuccessful. Twenty clones were randomly isolated and injected intravenously. Although some clones appeared to be quantitatively more metastatic to bone than others, clonogenic cells were recovered from spines by day 20 in all cases. Subsequent analyses of overt tumor growth have been done using clones 2 and 13 of 4T1 (4T1.2 and 4T1.13). The data for clone 2 are presented in the 1999 Clinical & Experimental Metastasis paper. H&E stains of sections of spine from 4T1.2 tumor bearing mice are shown the 1999 Clinical & Experimental Metastasis paper (Figures 2G & H) and in Figure 1C of the PTHrP manuscript (attached).

More recently, we have investigated the growth properties of the tumor lines after direct inoculation into bone, via the tibia. Cells (5000) were injected directly into the tibia and the extent of growth monitored after 14 days. As expected, 4T1.13 cells injected via this route proliferate well (Figure 2D of the PTHrP manuscript). Intriguingly, we also found extensive growth of 66cl4 tumor cells in bone 18 days after intratibial injection (Figure 3D of PTHrP manuscript). Thus, 66cl4 cells have the capacity to grow in bone, but do not home to bone to the same extent as the 4T1.13. It is still unclear

why the 66cl4 line does not form bone tumors after inoculation into the mammary pad, since circulating 66cl4 cells are detected in the bone environment. (Figure 3C of PTHrP manuscript – please note the different scales of the y axis between Figures 3Cand 3D). They may fail to adhere to the endothelial cells lining the bone microvasculature or may be unable to extravasate into the bone marrow microenvironment. Further study using this model should provide interesting insights into the mechanism of this difference in metastatic ability.

Task 3. Identification of the factors that are required for metastasis to bone, both by analysis of candidate genes and by genome wide screening for novel genes required for metastasis to bone.

We are using two approaches to identify genes differentially expressed between 66cl4 and 4T1.2, i.e. between the two clones that are fully metastatic but with only one capable of metastasizing to bone. The two approaches are (a) candidate gene analysis and (b) cDNA microarray analysis.

(a) Candidate gene approach.
A number of genes have been implicated in breast cancer metastasis. These include parathyroid hormone related protein (PTHrP), and its receptor, estrogen receptor, IL6, IL11, TGFβ, VCAM-1, several integrins, plasminogen activator (uPA) and other proteinases, and osteopontin. So far, we have tested for expression of PTHrP, for expression and activity of several proteinases including the matrix metalloproteases MMP2 and MMP9, uPA and tPA, and expression of cell adhesion molecules such as the integrins beta3, alpha$_v$, alpha6 and the adhesion molecule VCAM-1.

Parathyroid hormone related protein.
PTHrP shares structural and functional homology with parathyroid hormone, including the mobilization of calcium via increased bone resorption (Evely, Bonomo et al. 1991) (Suva, Winslow et al. 1987) and has been implicated in the tendency of breast tumor cells to grow in bone (Guise, Yin et al. 1996) (Mundy 1991) (Southby, Kissin et al. 1990). However, recent clinical studies indicate that PTHrP expression in primary breast tumors predicts a more favorable prognosis and a reduced tendency to metastasize to bone (Henderson, Danks et al. 2001). We therefore investigated the role of PTHrP in our model of breast cancer metastasis to bone. The results are presented in detail in the accompanying manuscript which is being submitted for publication. In summary, we found the following:

- *In vitro* secretion of PTHrP varies considerably between cell lines, but does not correlate completely with bone metastatic capacity (see Figure 1A of PTHrP manuscript). Whilst secretion in one bone metastasising clone, 4T1.2 is higher than in the lung metastasising clone, 66cl4 and in the non-metastatic 67NR clone, secretion of PTHrP in another bone metastasising clone, 4T1.13, was not detectable.

- *In situ* PTHrP expression by tumors derived from these cell lines does not correlate with *in vitro* secretion levels, suggesting that tumor cell PTHrP expression is regulated by the host microenvironment. Primary tumors derived from 67NR, 66cl4, 4T1.2 and 4T1.13 express similar amounts of PTHrP as assessed by indirect immunohistochemistry (IHC) (not shown). PTHrP expression is also strong in bone metastases (Figure 1C of PTHrP manuscript).

- Overexpression of PTHrP in the bone metastasising 4T1.13 clone, that does not normally secrete PTHrP *in vitro*, does not enhance the ability of the tumor cells to metastasize from the mammary gland to bone (Figures 2A and B of the PTHrP manuscript). In this experiment,

expression of an antisense PTHrP construct in 4T1.13 tumor cells significantly reduced tumor burden in bone (Figure 2B of PTHrP manuscript); however the slower *in vitro* growth rate (Figure 2C of PTHrP manuscript) and reduced primary tumor growth rate of the these tumor cells (not shown) could explain the reduced bone metastatic tumor burden. Direct injection into the tibia of 4T1.13 expressing either sense or antisense constructs for PTHrP did not affect tumor cell growth (Figure 2D of the PTHrP manuscript).

- The lung metastasizing clone, 66cl4, does not form overt tumors in bone after injection into the mammary gland, but grows rapidly in bone if deposited there directly by intra-tibial injection (Figure 3D of manuscript). Thus its inability to metastasize to bone relates more to a homing or invasion defect rather than an inability to proliferate in a bone environment.

- Expression of PTHrP in 66cl4 did not enhance bone tumor metastasis after inoculation of the cells into the mammary gland (Fig 3C of PTHrP manuscript) or affect the growth of 66cl4 in bone after direct intratibial injection. (Fig 3D of PTHrP manuscript).

A summary of PTHrP expression and metastatic properties of the various lines is included in Table 1.

Cell line	**In Vitro PTHrP Secretion (pmol/l/mg protein)**	**Site of inoculation**	**Bone Metastases** (visible by histology)	**Lung Metastases**
67NR	1.8	Mammary gland Tibia Left ventricle	No nd Yes	No nd Few
66cl4	2.0	Mammary gland Tibia Left ventricle	No Yes Yes	Few No Yes
66cl4PTHrP	160	Mammary gland Tibia Left ventricle	No Yes nd	Few No nd
4T1.13	2.0	Mammary gland Tibia Left ventricle	Yes Yes Yes	Yes No nd
4T1.13PTHrP	15.0	Mammary gland Tibia Left ventricle	Yes Yes Yes	Yes No nd
4T1.13PTHrP antisense	2.0	Mammary gland Tibia Left ventricle	Yes Yes Yes	Yes No nd

Table 1. Effect of PTHrP expression on metastasis to lung and bone. (nd: not determined)

Proteinases

The matrix metalloproteinase (MMP) and plasminogen activator (PA) families have been linked to the ability of breast tumor cells to metastasize (Andreasen, Kjoller et al. 1997) (Ornstein, MacNab et al. 1999) (Pulyaeva, Bueno et al. 1997). In collaboration with Drs. Rik Thompson and E. Allan at St. Vincent's Institute of Medical Research, we have determined the activity profiles of MMP2, MMP9, uPA and tPA for the three lines, 67NR, 66cl4 and 4T1.2 (Table 2). The 4T1.2 line has high levels of uPA and MMP9, moderate levels of tPA and the ability to activate MMP2. The 66cl4 line exhibits

low to moderate levels of these activities and low to negligible amounts are detected in 67NR. These activities are consistent with their metastatic capacities and with their invasive potential using *in vitro* invasion assays. Interestingly, tPA activity was similar in all three lines. The data on the MMP studies are in press in Clinical and Experimental Metastasis. A copy of the galley proofs is attached.

We have generated retroviral expression constructs for uPA in both sense and antisense orientation to express in the cells. It is our intention to either increase or decrease uPA activity in the lines, as appropriate, and determine the effect of this alteration on the metastatic capacity of the tumors.

Protease/ Activity	**Cell Line**		
	67NR (non-met)	**66cl4 (lungs)**	**4T1.2 (lungs and bone)**
MMP2 activation	+/-	+	+++
MMP9	-	+	+++
uPA	-	+	+++
tPA	+	+	+

Table 2. Proteinase activity in lines with differing metastatic potentials

Adhesion molecules

Adhesion molecules play an important role in tumor metastasis by specifying sites for vascular endothelial cell adhesion. Particular combinations of integrin alpha and beta subunits have been implicated in metastases, for example $\alpha_4\beta_1$, in homing of leucocytes to bone and $\alpha_v\beta_3$, in melanoma metastasis, while other combinations specify binding to different cell types (Rajotte, Arap et al. 1998). Integrin $\alpha_v\beta_3$ binds fibronectin, vitronectin, von Willibrand factor and osteopontin. Mice null for β_3 expression have non-functional osteoclasts and an osteopetrotic phenotype (McHugh, Hodivala-Dilke et al. 2000). Vascular cell adhesion molecule-1 (VCAM-1) expression by the endothelium is required for homing of hemopoietic progenitor cells to the bone marrow after transplantation (Frenette, Subbarao et al. 1998). To investigate whether these adhesion molecules play a role in specifying the ability to metastasize to bone, the expression of integrins β_1, β_3, α_v and α_6 and the adhesion molecule VCAM-1 has been measured in 67NR, 66cl4 and 4T1.2. Differential expression was only seen for β_3 and VCAM-1 (Figure 3), suggesting that these adhesion molecules may be important factors in enabling circulating 4T1.2 tumor cells to attach to the vascular endothelium of bone. The significance of these observations is being investigated by gain or loss of function experiments. We have prepared retroviral expression constructs for integrin β3 and selected for cells with increased or decreased expression by FACS. Expression of integrin β3 enhances the *in vitro* migration of the 66cl4 cell line. As shown in the 2001 Clinical & Experimental Metastasis (CEM) paper, 4T1.2 cells have a greater ability to migrate in response to a chemotactic signal (Figure 4 of the CEM paper). Expression of integrin β3 in 66cl4 cells confers on them a migratory capacity similar to that of 4T1.2 cells (Figure 4).

The modified lines will be injected into mice for analysis of distribution of metastases.

(b) cDNA microarrays

An array containing 5000 human genes was used to screen for differences in gene expression between RNA isolated from tumors derived from 66cl4 and 4T1.2. The array was competitively hybridized with Cy3 (green) labelled 66cl4 and Cy5 (red) labelled 4T1.2 cDNA. Genes over-expressed in 66cl4 appear green and those over-expressed in 4T1.2 appear red. Genes with equivalent expression in the two lines appear yellow. Approximately 40 differentially expressed genes were identified.

Differential expression was been further tested by northern analysis for nine of these genes and confirmed in four (Figure 5). This indicates the importance of confirming the microarray results using an independent technique. The four genes for which differential expression has been confirmed are osteopontin (OPN), caveolin-1 (Cav-1), bone morphogenic protein-4 (BMP-4) and protease nexin-1 (PN-1). Differential expression of the remaining 31 genes identified in the initial array screen remains to be tested by northern and/or RT-PCR analysis. Very soon we will have access to a 15k mouse cDNA array and we intend to screen the two lines again for further expression differences.

Caveolin-1

Caveolin-1 is the major structural component of membrane invaginations called caveolae (Okamoto, Schnegel et al. 1998). It interacts with a number of signalling molecules including H-ras, Src family tyrosine kinases, eNOS and EGFR and appears to down-regulate signalling via the p42/44 MAPK pathway (Galbiati, Volonte et al. 1998). Down-regulation of caveolin-1 expression has been shown to cause transformation of cells *in vitro*, leading to the suggestion that caveolin-1 may function as a tumor suppressor (Lee, Reimer et al. 1998). In our model of metastasis, caveolin-1 is down-regulated in the lymph node, lung and bone metastasizing clone, 4T1.2, compared to 66cl4 that metastasizes only to lymph nodes and lung, leading us to hypothesize that it is a bone metastasis suppressor gene.

Whilst northern blot analysis of RNA from tumors shows elevated caveolin-1 in 66cl4, tissue culture cells shows little difference in caveolin-1 expression between 4T1.2 and 66cl4 (data not shown). Thus, caveolin-1 expression is regulated *in vivo* by factors not present *in vitro*. This result illustrates the importance of the microenvironment on gene expression and therefore the advantages of using an *in vivo* model system. Differential expression of caveolin-1 in tumor derived material was also confirmed by western blot analysis (not shown), immunohistochemistry (Figure 6) and by *in situ* hybridization (Figure 7).

Expression of caveolin-1 was recently measured by immunohistochemistry on a tissue array of approximately 400 human breast tumors for which clinical outcome data is available. This work has been done in collaboration with Dr. O. Kallioniemi (NHGRI/NIH). Whilst many primary human breast tumors do not express caveolin-1, a subset that do express this gene appear to display longer survival. A detailed statistical analysis of these data is underway.

Experiments aimed at altering caveolin-1 expression in the mtastasis model are underway. We have obtained a dominant negative form of caveolin-3 from Dr. J. Hancock (Roy, Luetterforst et al. 1999) as an alternative way of blocking caveolin-1 function. This construct blocks caveolin-1 function (J. Hancock: personal communication).

A manuscript describing the role of caveolin-1 in metastasis is in preparation.

Protease nexin-1

Protease nexin I (PN-I) is a soluble, broad spectrum protease inhibitor (Akaaboune, Hantai et al. 1998) expressed in various cell types including bone cells and has been implicated in glioma tumorigenesis (Rao, Baker et al. 1990). PN-1 is expressed at higher levels in tumors from the bone metastasizing clone, as assessed by in situ hybridisation (Figure 8).

PN-1 has been cloned into a retroviral expression vector and screening for increased expression in 66cl4 is underway.

Osteopontin

Of the four genes identified, only the bone matrix protein osteopontin has previously been implicated in tumor metastasis, although not in metastasis to a specific organ (Schwirzke, Schiemann et al. 1999). Interestingly, we find decreased osteopontin mRNA levels in the more aggressively metastatic 4T1.2 line compared to 66cl4. This observation will be investigated further.

Bone morphometric protein-4

BMP-4 regulates development of skeletal structures by inducing differentiation of osteoblasts from mesenchymal precursors and by mediating apoptosis of the neural crest (Ahrens, Ankenbauer et al. 1993). BMP-4 is expressed at lower levels in the bone metastasizing clone. We have not yet investigated the role of this gene in metastasis.

FIGURE LEGENDS

Figure 1

Metastasis pattern of the 4T1.13 tumor after inoculation into the mammary gland. Tumor cells, (1×10^5) were inoculated into the mammary gland. After 25 days the mice were culled and organs removed for analysis of metastatic burden by RTQ-PCR.

Figure 2

Kinetics of metastasis of 4T1.13 to lung and bone. 4T1.13 cells were inoculated as described for Figure 1. Mice were harvested at weekly intervals beginning 14 days after inoculation. The lungs and spine were removed for analysis of tumor burden by RTQ-PCR.

Figure 3

FACS profiles of β_3 and α_v integrins (A) and VCAM-1 and $\alpha_4\beta_1$ integrin (B) expression in the tumor lines. Cells were incubated with the relevant primary antibody for 20 min on ice, washed then incubated with FITC-conjugated secondary antibody for 20 min prior to FACS analysis. The filled in red profile shows the fluorescence from use of the secondary antibody alone.

Figure 4

Stable transfection of the beta3 integrin increases migration of 66cl4 cells. Membranes with 8 micron pores were coated with 5ug/ml gelatin. 2×10^5 cells in 200ul alpha-MEM were placed in the top compartment and allowed to migrate towards alpha-MEM plus 10% FCS in the bottom compartment for 6 hours. Cells on the lower side of the membranewere stained and counted (examples of stained cells on lower side of membrane are shown).

Figure 5

Northern analysis of 4 genes found to be differentially expressed on the cDNA microarray. RNA was isolated from both tissue culture cells and tumors of the two bone metastasising clones, 4T1.2 and 4T1.13, from the lung metastasising clone 66cl4, from the non-metastatic 67NR line and from normal mammary gland (fat pad). The membrane was hybridized with probes from caveolin-1, protease nexin-1, bone morphometric protein 4, osteopontin and heat shock protein 73 as a housekeeping gene. Note the differences in mRNA expression between tissue culture and tumors from the same cell line.

Figure 6

Immunohistochemical detection of caveolin-1 expression in primary tumors. A polyclonal anti-caveolin-1 antibody was hybridised to sections from 4T1.2 and 66cl4 primary tumors. A biotin-conjugated secondary antibody was used to detect the primary antibody and visualised using streptavidin-peroxidase and a color reaction. Non-immune (N.I.): rabbit antiserum was used as a control.

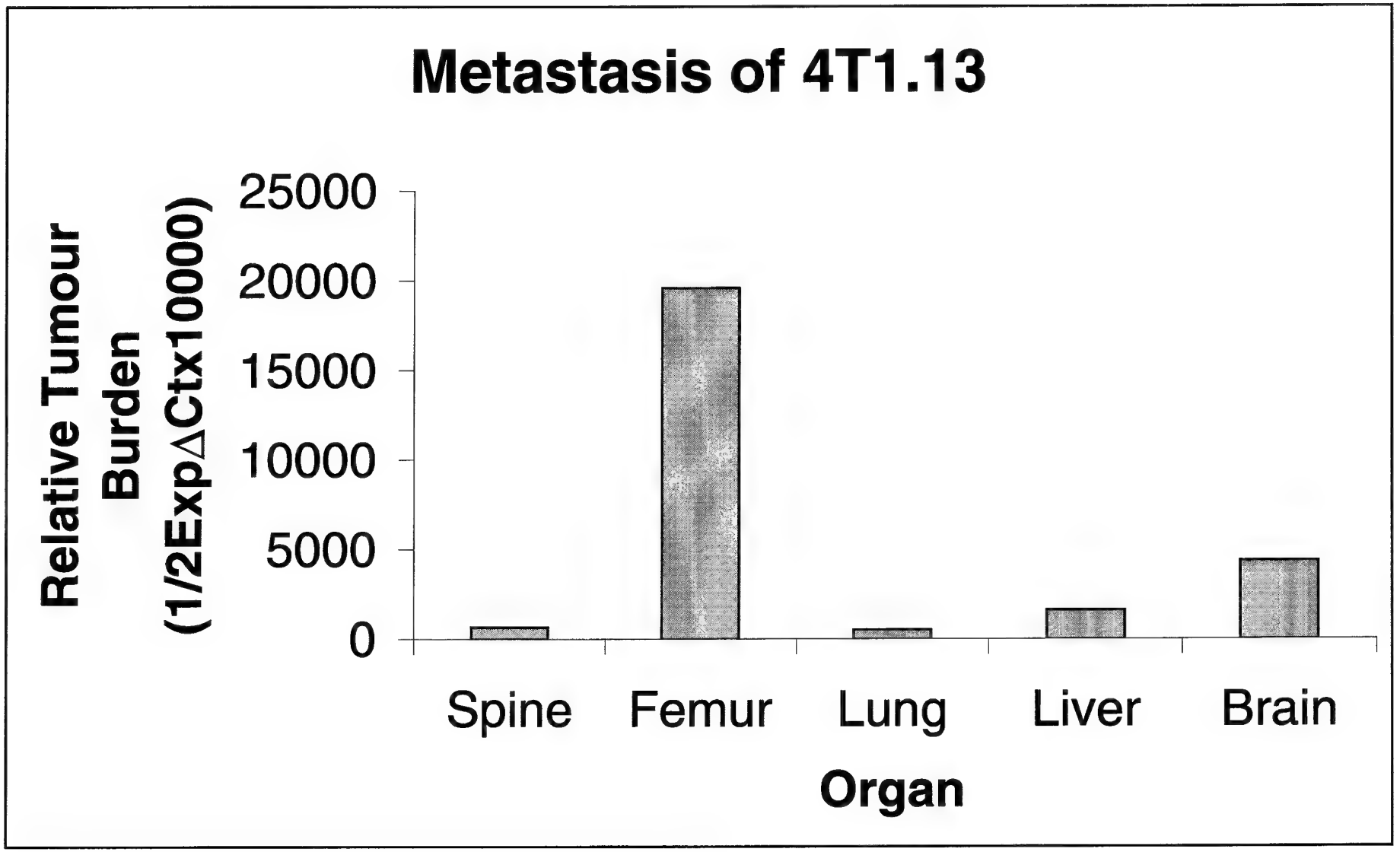
Figure 7

In situ hybridisation of caveolin-1 in primary tumors. Antisense and sense DIG labelled riboprobes were hybridised to sections cut from primary tumors of 4T1.2 and 66cl4. An alkaline phosphatase conjugated anti-DIG antibody was used to detect the riboprobes.

Figure 8

In situ hybridisation of protease nexin 1 in primary tumors. Antisense and sense DIG labelled riboprobes were hybridised to sections cut from primary tumors of 4T1.2 and 66cl4. An alkaline phosphatase conjugated anti-DIG antibody was used to detect the riboprobes.

Fig. 1


Metastasis of 4T1.13
Relative Tumour Burden (1/2ExpΔCtx10000)
25000
20000
15000
10000
5000
0
Spine
Femur
Lung
Liver
Brain
Organ

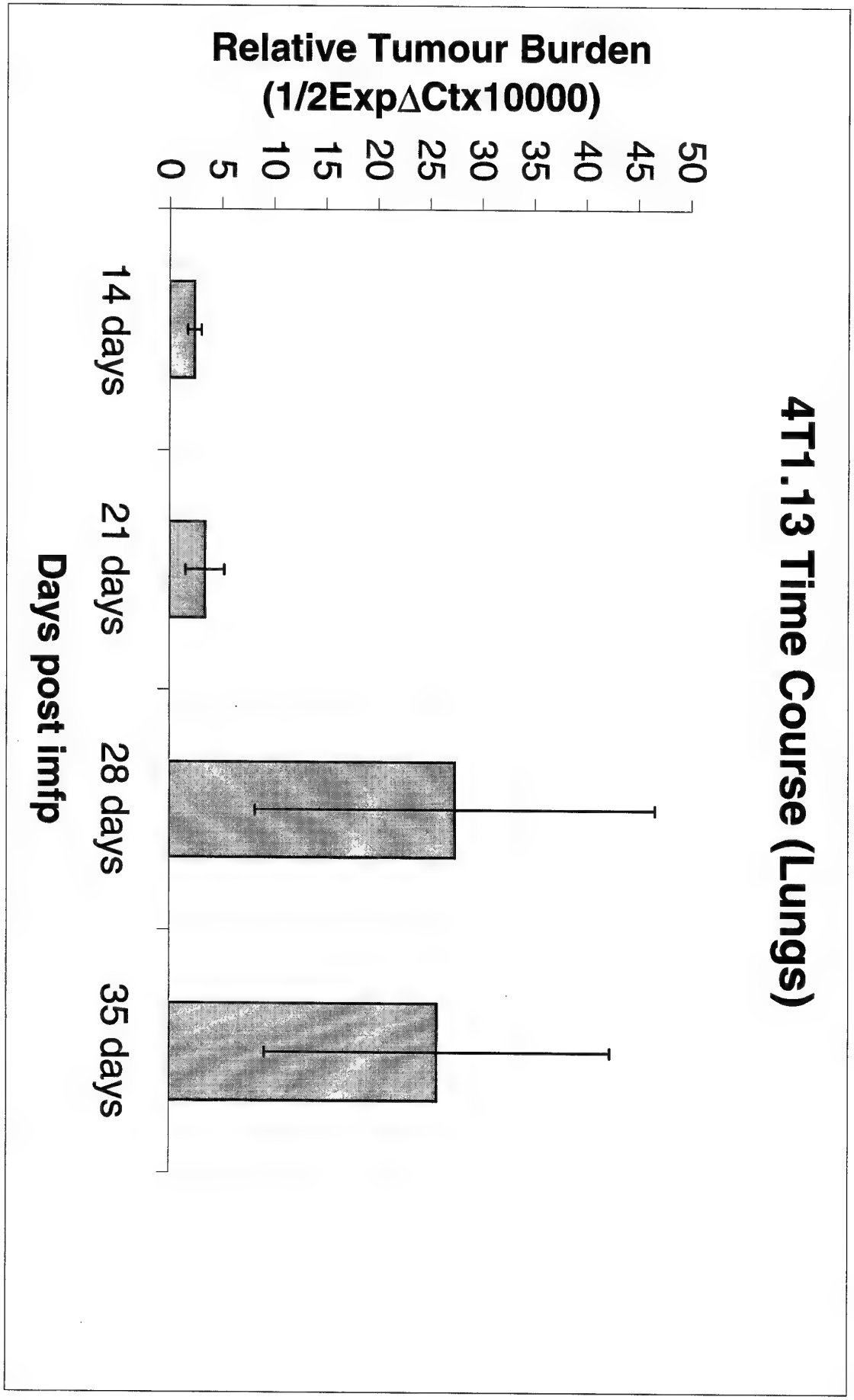

4T1.13 Time Course (Lungs)
Relative Tumour Burden
(1/2ExpΔCtx10000)
0
5
10
15
20
25
30
35
40
45
50
14 days
21 days
28 days
35 days
Days post imfp

Fig. 2A

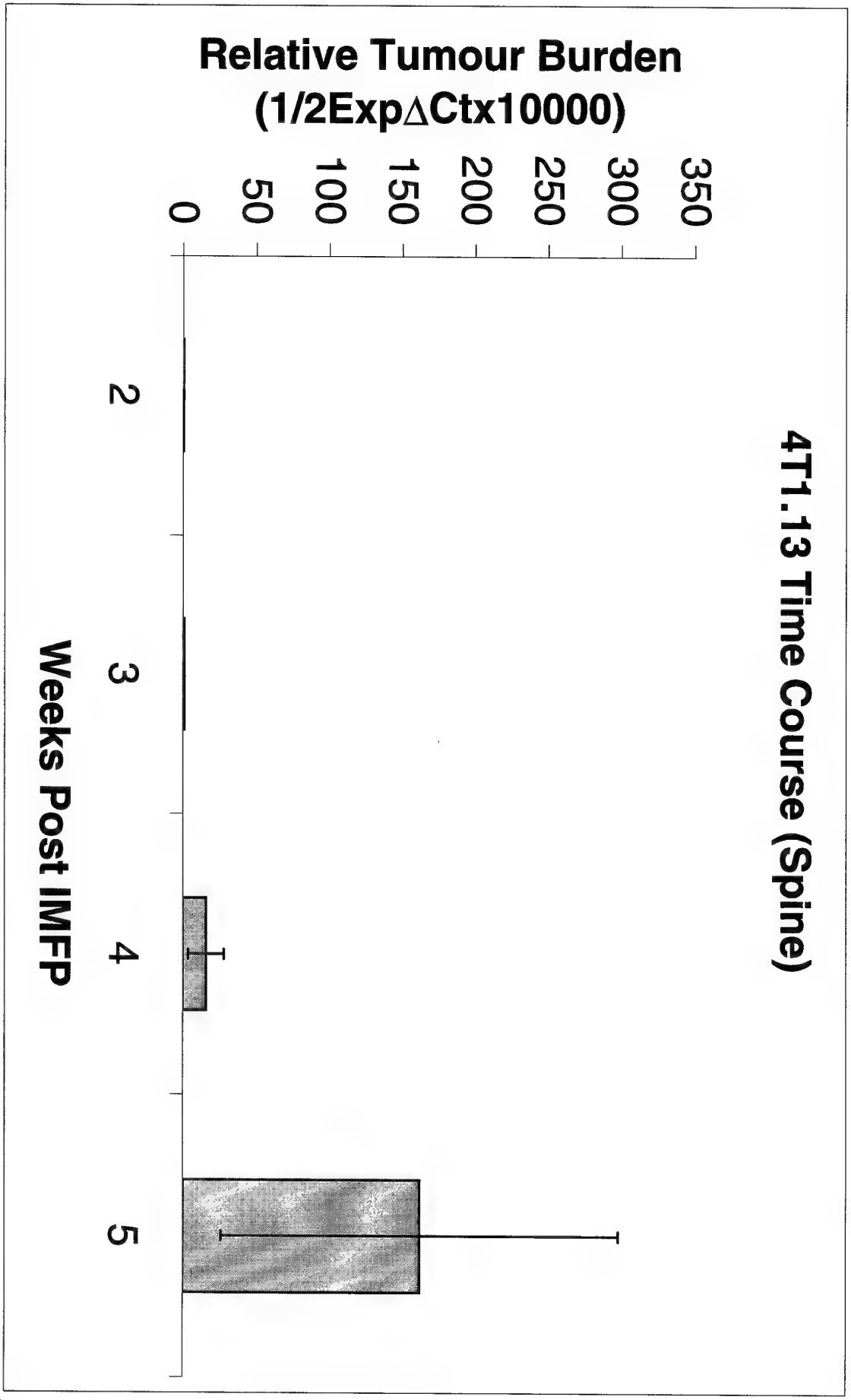
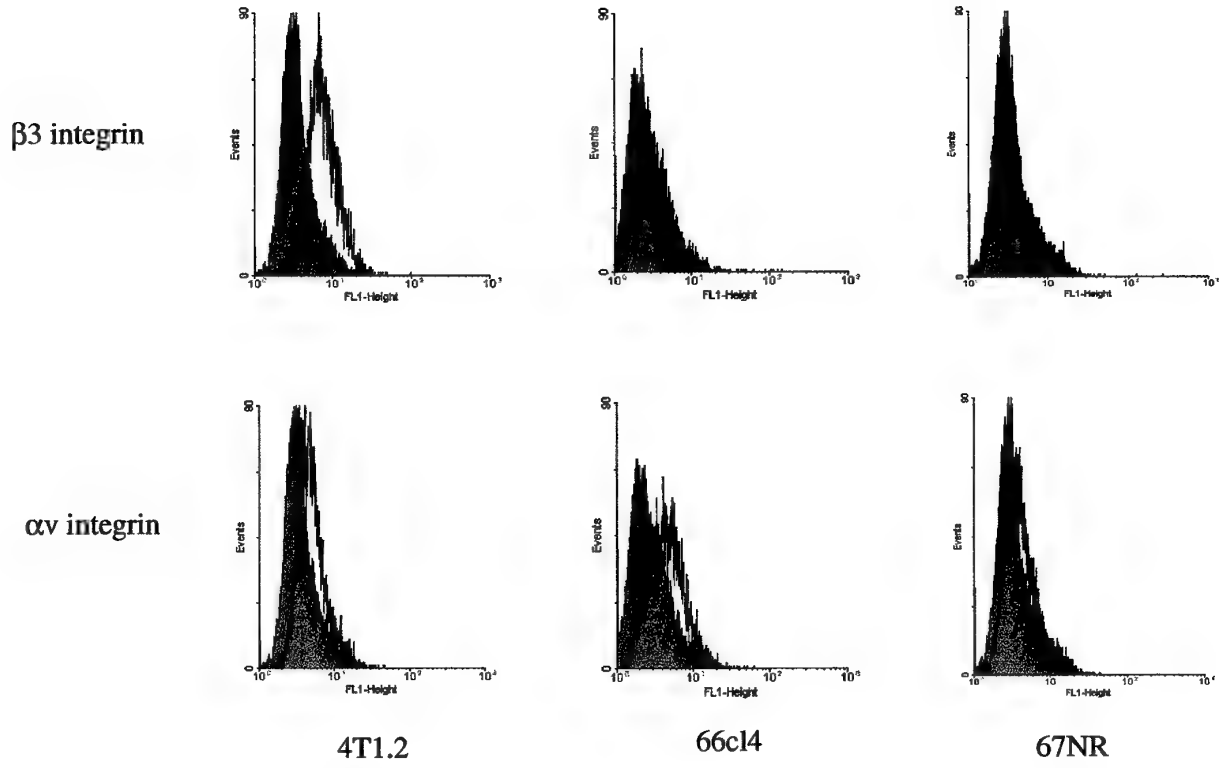

4T1.13 Time Course (Spine)
Relative Tumour Burden
(1/2ExpΔCtx10000)
0
50
100
150
200
250
300
350
2
3
4
5
Weeks Post IMFP

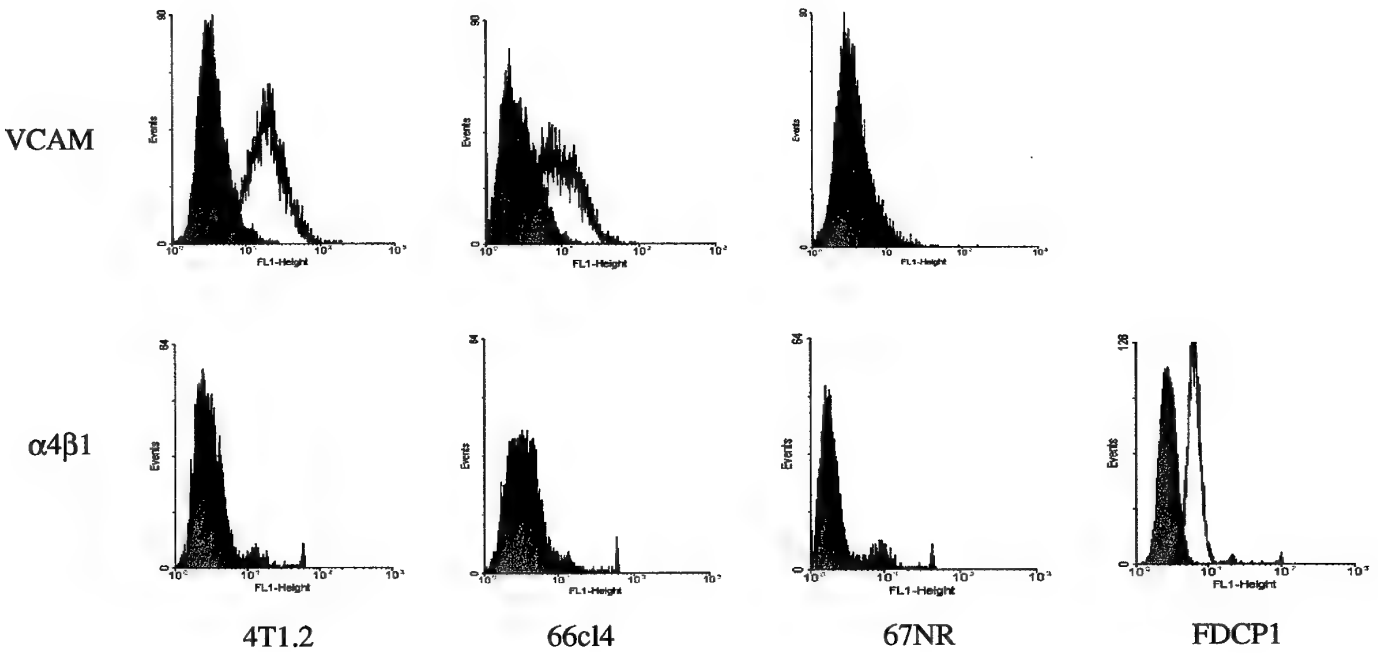
Fig. 2B

Fig. 3A



Expression of αvβ3 integrin on mouse cell lines

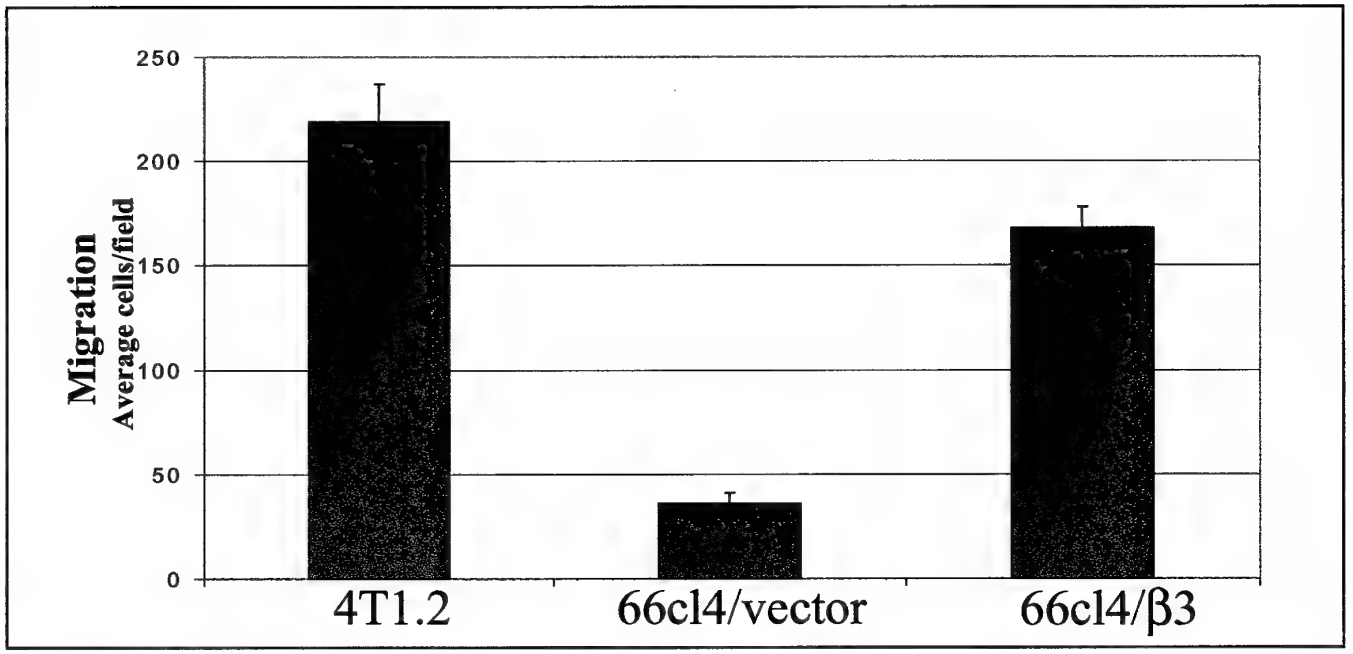
Fig. 3B

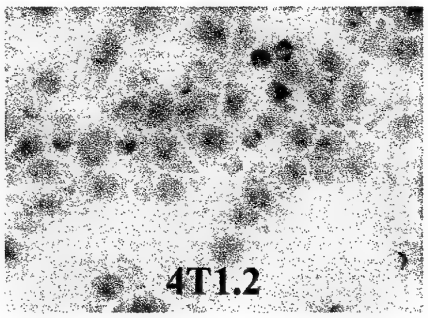


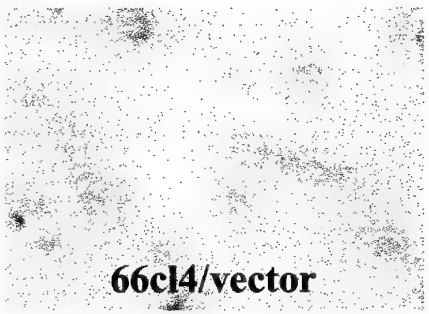
VCAM, but not α4β1 integrin, is expressed by the metastatic cell lines

Fig. 4

β3 integrin enhances *in vitro* migration of 66cl4







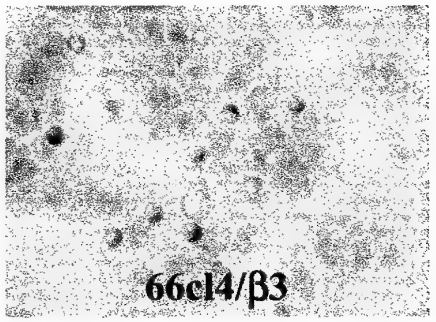
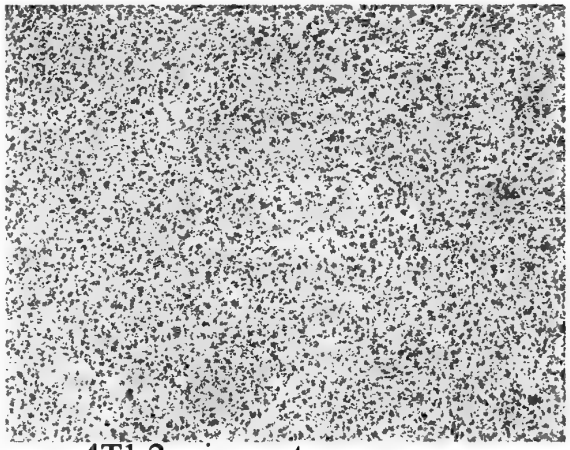


Fig. 5

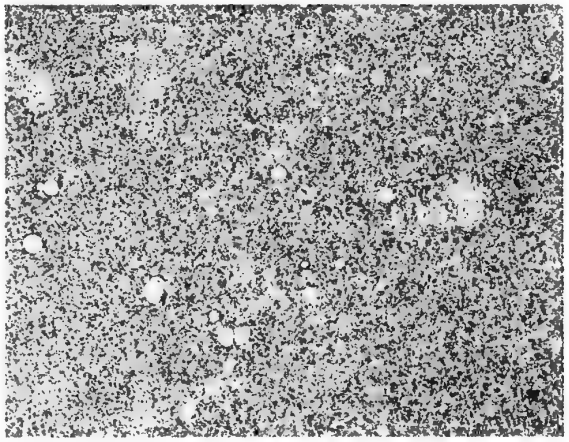
tissue culture cells
primary tumours
bone metastases
lung metastases
4T1.13
4T1.2
66cl4
67NR
4T1.13
4T1.2
66cl4
67NR
tatpad
cav-1
PN I
BMP 4
OPN
hsc 73

Fig. 6

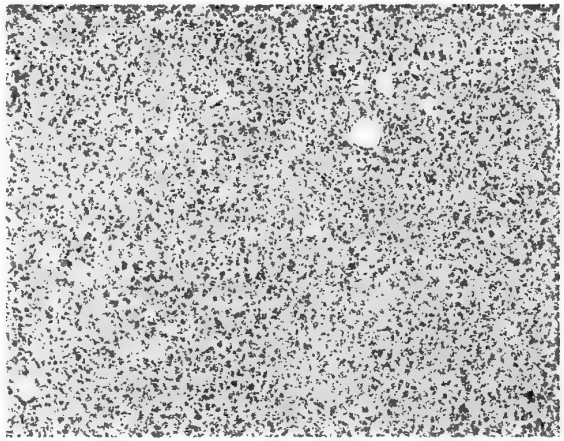
IHC: anti-caveolin polyclonal antibody, 1/100 dilution



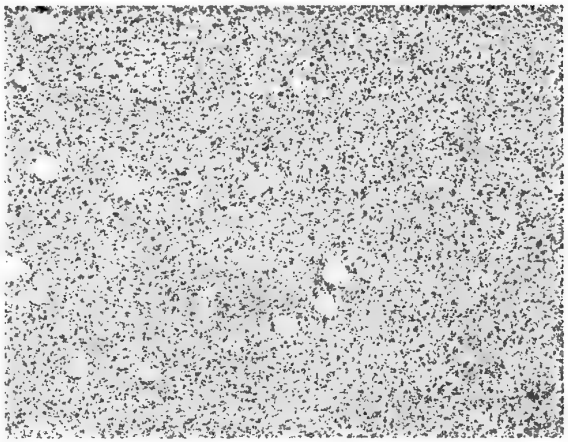
4T1.2 primary tumour
rabbit anti-caveolin polyclonal



66cl4 primary tumour
rabbit anti-caveolin polyclonal



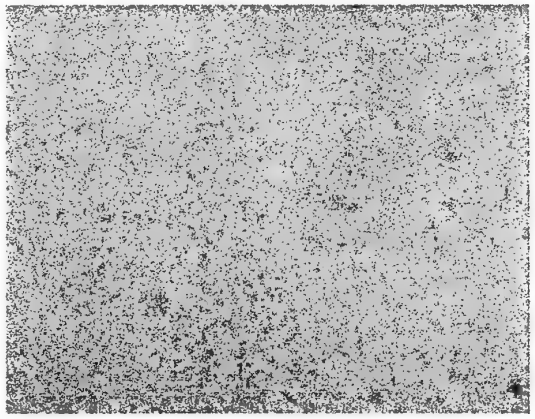
4T1.2 primary tumour
non-immune rabbit serum



66cl4 primary tumour
non-immune rabbit serum

Fig. 7

Caveolin-1 ISH



4T1.2 antisense

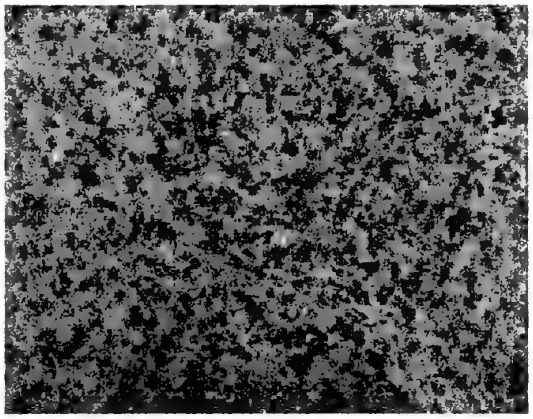
66cl4 antisense

4T1.2 sense

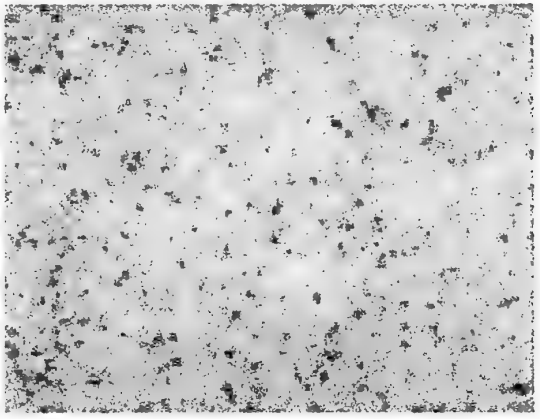
66cl4 sense

Fig. 8

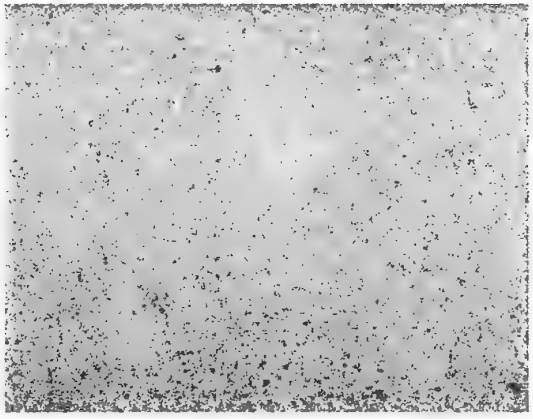
Protease nexin I IHS



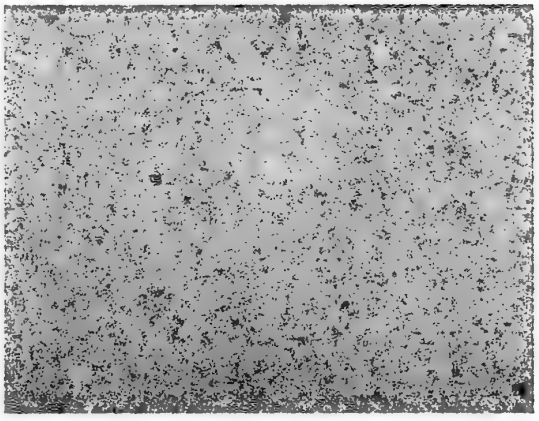
4T1.2 antisense



66cl4 antisense



4T1.2 sense



66cl4 sense

KEY RESEARCH ACCOMPLISHMENTS

- The development and characterization of the only murine model of spontaneous metastasis from the mammary gland to bone. This model offers great potential in testing novel therapeutics targeted at bone metastases.

- Characterization of clones derived from the original carcinoma that have differing metastatic potentials.

- Development of a quantitative PCR technique to measure tumor burden in different tissues.

- The discovery by cDNA microarray analysis of a number of genes that have the potential to regulate metastasis from breast to bone.

- Demonstration that PTHrP expression may affect primary tumor growth, but its expression does not correlate with the extent of tumor growth within bone environment. PTHrP is therefore not a useful marker of bone metastasis. These data imply that the ability of tumor cells to metastasize to bone may be determined by cell adhesion and/or invasion properties rather than growth factors.

- Demonstration that increased levels of uPA activity, MMP9 activity and MMP2 activation correlate with the ability to metastasize to bone.

- Demonstration that β3 integrin and VCAM-1 levels are elevated in the bone metastasising clone.

- Proposal that caveolin-1, protease nexin 1 or osteopontin may be important in breast cancer metastasis to bone.

- A metastasis model that provides an excellent system for preclinical drug testing of anti-angiogenic and anti-metastatic drugs.

REPORTABLE OUTCOMES

- Manuscript published in *Clinical and Experimental Metastasis.*
  Lelekakis, M., Moseley, J.M., Martin, T.J., Hards, D., Williams, E., Ho, P., Lowen, D., Javni, J., Miller, F.R., Slavin, J., Anderson, R.L. (1999) A novel orthotopic model of breast cancer metastasis to bone. Clin. Exp. Metastasis 17: 163-170.

- Manuscript in press in *Clinical & Experimental Metastasis*:
- Tester, A.M., Ruangpanit, N., Anderson, R.L. and Thompson, E.W. (2001) MMP-9 secretion and MMP-2 activation distinguish invasive and metastatic sublines of a mouse mammary carcinoma system showing epithelial-mesynchymal transition traits.

  Tavaria, M.D. et al (2001) Parathyroid hormone related protein (PTHrP) expression does not dictate the ability of breast carcinoma lines to metastasise to bone (manuscript in preparation).

  Sloan, E. et al (2001) The role of caveolin-1 in breast cancer progression (manuscript in preparation).

- Abstract and poster presentation to the 7th International Congress of the Metastasis Research Society, 1998.
  A Model of spontaneous breast cancer metastasis to bone. Anderson, R.L., Lelekakis, M., Miller, F., Williams, E.D., Hards, D., Martin, T.J. and Moseley, J.M.

- Abstract, poster and platform presentation to the Keystone Symposium entitled Molecular Pathogenesis of Bone Disease, California, 1999.
  Characterization of a novel murine model of breast cancer metastasis to bone. Tavaria, M., Sloan, E., Lelekakis, M., Ho, P., Hards, D., Williams, E., Martin, T.J., Moseley, J. and Anderson, R.L.

- Keystone travel grant awarded to M. Tavaria for the best short platform presentation.

- B. Med. Sc. degree awarded to Mr. Marcus Foo based on his thesis that investigated the relationship between the presence of metastatic cells in bone marrow and the development of overt bone tumors.

- Platform presentation to the DOD Era of Hope Meeting, 2000.
  Mechanisms of breast cancer metastasis to bone. Anderson, R.L., Lelekakis, M., Tavaria, M., Sloan, E., Martin, T.J., Hards, D., Ho, P., Moseley, J.M.

- Komen Dissertation Award to E. Sloan for salary support whilst investigating the function of some of the genes found by cDNA microarray analysis to be differentially expressed in the bone metastasising clone.

Platform presentation by Ms. E. Sloan to the 8th International Congress of the Metastasis Research Society, London, Sept, 2000. *Genes involved in breast cancer metastasis to bone*. *E Sloan, M Tavaria, M Lelekakis, J Moseley, R Anderson.

Poster presentation to AACR, 2000,
Tavaria,M.D., Lelekakis,M., Sloan,E., Ho,P., Hards,D., Martin,T.J., Moseley,J. and Anderson,R.L. *Factors important in breast cancer metastasis to bone*. 91st American Association for Cancer Research Annual Meeting (San Francisco, April 2000)

Abstract , poster and platform presentation:
Tavaria,M.D., Sloan,E., Lelekakis,M., Natoli, A., Moseley,J. and Anderson,R.L. *Characterisation of an orthotopic mouse model of breast cancer metastasis to bone*. 10th Annual Scientific Meeting of the Australian and New Zealand Bone and Mineral Society (Hamilton Island, Nov 2000)

Successful application to the NIH to extend and build upon the data obtained during the tenure of this award.

Successful application to The Wellcome Trust for funding to purchase a real time PCR system for this project (until now, we have been commuting to another institute to borrow their equipment).

CONCLUSIONS

The major achievement in the first year of this grant was the characterization of the murine breast cancer model of metastasis from the mammary gland to bone. To our knowledge, this is the only model that mimics the human disease and it provides a unique opportunity to search for the genes that control metastasis to bone. We have demonstrated that tumor clones derived from the 4T1 tumor line successfully metastasize to bone, but that other metastatic tumor lines derived from the original tumor do not, despite the presence of circulating tumor cells within the bone. Using intracardiac injections, we have shown that the non-metastatic clone can proliferate in bone when forced there by arterial blood flow and that the clone that metastases only to lung from the mammary gland can proliferate in bone after intra-tibial injection. These results imply that the 4T1 derived tumor lines have the unique ability to invade bone or that the other clones have lost this ability. The results suggest that cell adhesion molecules or proteases may be responsible for the differences in ability to metastasize to bone.

A substantial amount of effort has been put into the characterisation of genes that are differentially expressed between the bone metastasising clones, 4T1.2 and 4T1.13, and another fully metastatic line that cannot metastasize to bone, 66cl4. Our **candidate gene approach**, as outlined in the proposal, has focussed on genes implicated by others in breast cancer metastasis in general or to bone in particular. For PTHrP, we have demonstrated that, contrary to some published data, PTHrP does not drive metastatic growth in bone. This finding is interesting in the light of recent clinical data (Henderson et al, 2001) showing that patients with high PTHrP expression in their primary tumors have longer survival and fewer bone metastases. Taken together, these data bring into question the widely held belief that PTHrP expression is a poor prognostic indicator.

For a number of other candidate genes, we have demonstrated a correlation between increased expression and the ability to metastasize to bone. The relevance of these correlations awaits the outcome of gain or loss of function experiments, similar to those reported for PTHrP. The development of an RTQ-PCR assay to quantitatively measure metastatic tumor burden is an important development that will enable more efficient experimental analysis.

Our **genome wide screening** has revealed differential expression of a few genes not previously implicated in metastasis. Functional studies are underway with these genes to determine their importance in bone metastasis.

Ultimately, we will need to examine the expression of genes that appear in the mouse model to be important in bone metastasis, in primary human breast tumors from patients for whom clinical outcome is known. It is our belief that we will be able to identify genes whose expression is prognostic of subsequent metastatic behaviour of the tumor. For example, if the expression of a particular gene is linked to subsequent metastasis to bone, it will be possible to treat that subset of patients more aggressively to prevent bone metastasis or with drugs such as bisphosphonates that may reduce or eliminate bone disease. Further, a gene that directly regulates metastasis to bone may become the target for a new therapeutic strategy.

REFERENCES

Ahrens, M., T. Ankenbauer, et al. (1993). "Expression of human bone morphogenetic proteins-2 or -4 in murine mesenchymal progenitor C3H10T1/2 cells induces differentiation into distinct mesenchymal lineages." DNA Cell Biol. **12**: 871-880.

Akaaboune, M., D. Hantai, et al. (1998). "Developmental regulation of the serpin, protease nexin-1, localization during activity-dependent polyneuronal synapse elimination in mouse skeletal muscle." J. Comp. Neurol. **397**: 572-579.

Andreasen, P. A., J. Kjoller, et al. (1997). "The urokinase-type plasminogen activator system in cancer metastasis: a review." Int. J. Cancer **72**: 1-22.

Evely, R. S., A. Bonomo, et al. (1991). "Structural requirements for the action of parathyroid hormone-related protein (PTHrP) on bone resorption by isolated osteoclasts." J. Bone Mineral Res. **6**: 85-93.

Frenette, P. S., S. Subbarao, et al. (1998). "Endothelial selectins and vascular cell adhesion molecule-1 promote hematopoietic progenitor homing to bone marrow." Proc. Nat. Acad. Sci. USA **95**: 14423-14428.

Galbiati, F., D. Volonte, et al. (1998). "Targeted downregulation of caveolin-1 is sufficient to drive cell transformation and hyperactivate the p42/44 MAP kinase cascade." EMBO J. **17**: 6633-6648.

Guise, T. A., J. J. Yin, et al. (1996). "Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis." Journal of Clinical Investigation **98**: 1544-1549.

Henderson, M. A., J. A. Danks, et al. (2001). "Parathyroid hormone-related protein production by breast cancers, improved survival and reduced bone metastases." J. Natl. Cancer Inst. **93**: 234-237.

Lee, S. W., C. L. Reimer, et al. (1998). "Tumor cell growth inhibition by caveolin re-expression in human breast cancer cells." Oncogene **16**: 1391-1397.

McHugh, K. P., K. Hodivala-Dilke, et al. (2000). "Mice lacking beta3 integrins are osteosclerotic because of dysfunctional osteoclasts." J. Clin. Invest. **105**: 433-440.

Mundy, G. (1991). "Mechanisms of osteolytic bone destruction." Bone **12**: S1-S6.

Okamoto, T., A. Schnegel, et al. (1998). "Caveolins, a family of scaffolding proteins for organising "preassembled signaling complexes" at the plasma membrane." J. Biol. Chem. **273**: 5419-5422.

Ornstein, D. L., J. MacNab, et al. (1999). "Evidence for tumor-host cooperation in regulating MMP-2 expression in human colon cancer." Clin. Exp. Metastasis **17**: 205-212.

Pulyaeva, H., J. Bueno, et al. (1997). "MT1-MMP correlates with MMP-2 activation potential seen after epithelial to mesenchymal transition in human breast carcinoma cells." Clin. Exp. Metastasis **15**: 111-120.

Rajotte, D., W. Arap, et al. (1998). "Molecular heterogeneity of the vascular endothelium revealed by in vivo phage display." J. Clin. Invest. **102**: 430-437.

Rao, J. S., J. B. Baker, et al. (1990). "Serpin inhibitors of urokinase and thrombin in normal rat brain and the 9L brain tumor: evidence for elevated expression of protease nexin 1-like inhibitor and a novel sodium dodecyl sulphate-activated tumor antithrombin." Cancer Res. **50**: 5039-5044.

Roy, S., R. Luetterforst, et al. (1999). "Dominant-negative caveolin inhibits H-Ras function by disrupting cholesterol-rich plasma membrane domains." Nature Cell Biol. **1**: 98-105.

Schwirzke, M., S. Schiemann, et al. (1999). "New genes potentially involved in breast cancer metastasis." Anticancer Research **19**: 1801-1814.

Southby, J., M. W. Kissin, et al. (1990). "Immunohistochemical localisation of parathyroid hormone-related protein in human breast cancer." Cancer Research **50**: 7710-7716.

Suva, L. J., G. A. Winslow, et al. (1987). "A parathyroid hormone-related protein implicated in malignant hypercalcemia: cloning and expression." Science **237**: 893-896.

APPENDICES:

1. A copy of the article published in *Clinical and Experimental Metastasis.*

2. A copy of the manuscript accepted for publication in *Cancer Research.*

3. A copy of the PTHrP manuscript

Clinical & Experimental Metastasis **17:** 163–170, 1999.

A novel orthotopic model of breast cancer metastasis to bone

Maria Lelekakis[1], Jane M. Moseley[2], T. John Martin[2], Daphne Hards[2], Elizabeth Williams[2], Patricia Ho[2], Darren Lowen[1], Jeannie Javni[1], Fred R. Miller[3], John Slavin[4] & Robin L. Anderson[1]

[1]*Trescowthick Research Laboratories, Peter MacCallum Cancer Institute, Locked Bag # 1, A'Beckett St., Melbourne, Victoria 8006; Australia,* [2]*St. Vincent's Institute of Medical Research, 41 Victoria Parade, Fitzroy, Victoria 3065; Australia,* [3]*Karmanos Cancer Institute, 110 E. Warren Ave., Detroit, MI 48201, USA;* [4]*Department of Pathology, St. Vincent's Hospital, 41 Victoria Parade, Fitzroy, Victoria 3065, Australia*

Received 21 October 1998; accepted in revised form 19 January 1999

Key words: bone metastasis, breast cancer, model, PTHrP

Abstract

Breast cancer affects approximately one woman in twelve and kills more women than any other cancer. If detected early, patients have a five year survival rate of 66%, but once metastatic disease has developed, there is no effective treatment. About 70% of patients with metastatic disease have bone involvement, while lungs and liver are the other common targets. Bone metastases cause severe pain, pathological fractures and hypercalcaemia and thus are a significant clinical problem. The development of new therapies for metastatic breast carcinoma depends on a better understanding of the mechanism of homing of the tumour cells to bone, liver and lungs and the factors required for their growth in these organs. Research on mechanisms of breast cancer metastasis, particularly to bone, has relied on *in vitro* studies or on tumour models in which the inoculation route is designed to promote delivery of tumour cells to a specific organ. Metastases in bone are achieved by inoculation into the right ventricle of the heart. To our knowledge there has been no report of a model of metastatic spread from the mammary gland to distant sites which reliably includes bone. In this paper, we describe our recent development of a novel murine model of metastatic breast carcinoma. The new model is unique in that the pattern of metastatic spread closely resembles that observed in human breast cancer. In particular, these murine breast tumours metastasise to bone from the primary breast site and cause hypercalcaemia, characteristics not normally found in murine tumours, but common in human disease. Furthermore, in a preliminary characterisation of this model, we show that secretion of parathyroid hormone-related protein, a role for which has been implicated in breast cancer spread to bone, correlates with metastasis to bone. This model therefore provides an excellent experimental system in which to investigate the factors that control metastatic spread of breast cancer to specific sites, particularly bone. The special advantage of this system is that it involves the whole metastasis process, beginning from the primary site. Existing models consider mechanisms that pertain to growth of tumour once the site has been reached. An understanding of the regulation of these factors by potential therapeutic agents could lead to improvement in therapies designed to combat metastatic disease. For the first time, this development will allow exploration of the molecular basis of site-specific metastasis of breast cancer to bone in a clinically relevant model.

Introduction

Bone metastases are a major cause of morbidity in breast cancer and occur in 70% of patients who develop metastatic disease. Hypercalcaemia is a common complication due to excessive bone resorption which is caused by increased numbers of active osteoclasts. This leads to the breast cancer patient suffering severe pain and bone fractures [1]. The preference of breast tumour cells for growth in bone is not fully understood, but is undoubtedly facilitated by their ability to stimulate adjacent osteoclasts to resorb bone. This process is promoted by the ability of these tumour cells to express bone compatible matrix proteins and adhesion molecules and by tumour cell interactions with local growth factors in bone. Several factors that have normal roles in bone turnover and metabolism have been detected in primary breast cancers. These include parathyroid hormone-related protein (PTHrP), tumour necrosis factor-α (TNFα), transforming growth factor-β (TGFβ) and prostaglandins [1] [2], interleukin 6 (IL6) [3], interleukin 11 (IL11) [4], osteopontin [5] [6] and bone sialoprotein [6]. The ability of these factors to alter bone turnover and to be regulated in the bone environment may facilitate establishment of tumours in bone.

The development of animal models of human tumour cells injected into immunodeficient mice for the study of breast cancer spread to bone has provided strong evidence for the importance of PTHrP in the establishment of bone metastases [7]. However, these models are dependent upon

Correspondence to: Robin L. Anderson, Trescowthick Research Laboratories, Peter MacCallum Cancer Institute, Locked Bag # 1, A'Beckett St., Melbourne, Victoria, Australia, 8006. Tel: +61-3-9656-1284; Fax: +61-3-9656-1411

the injection of tumour cells into the left ventricle of the heart in order to promote tumour cell delivery to bone and therefore can only evaluate factors important for invasion of bone and interaction of tumour cells with the bone environment. Until now, no models of spontaneous metastasis of breast tumour cells from the primary site to bone have been reported, although orthotopic inoculation has been shown to result in development of secondary tumours in lymph nodes and lungs [8]. Breast cancer cells can be forced to the site of interest, for example by intravenous injection to promote growth in lungs, into the portal vein to produce liver tumours, and into the carotid artery to induce brain tumours [8]. To induce tumour cell growth in bone, cells can be injected directly into the tibia [9] or into the arterial system [10]. However, all of these routes result in a bolus of cells arriving in bone or other organ of interest, rather than the situation that occurs in clinical disease, in which cells escape gradually over time from the primary tumour and circulate through the vascular system or lymphatics before colonizing a distant organ.

We report here the development of a novel model of metastasis from the mammary gland to bone using a clonal tumour line derived from a spontaneously arising mammary tumour in a BALB/cfC3H mouse [11]. The isolation of sublines that are either non-metastatic, or that metastasise to lungs or that metastasise to lungs and liver has been described previously [12]. Here we report that one of the subpopulations, 4T1, also metastasises to bone. We have further characterised a single cell clone derived from 4T1 (4T1.2) that metastasises to bone and lung. The model is unique in that the pattern of metastatic spread closely resembles that observed in human breast cancer, causing osteolytic lesions in bone. Given the documented role of PTHrP in bone lesions of human breast cancer, we have examined the expression patterns of PTHrP both *in vitro* and *in vivo* and have found that both primary tumours and bone lesions of the bone metastasising clone express this growth factor.

Materials and methods

Cell culture

Four sublines of breast cancer cells, 67NR, 66cl4, 4T1 and 4T1.2, derived from a spontaneous carcinoma in a Balb/cfC3H mouse were used. The derivation of the first three of these sublines has been described previously [12]. Briefly, 67NR is a geneticin resistant variant derived from subpopulation 67, 66cl4 is a thioguanine/ouabain resistant variant of subpopulation 66 and 4T1 is a thioguanine resistant variant of subpopulation 410.4. Clone 4T1.2 was derived by single cell cloning of 4T1 (Figure 1).

The tumour lines were maintained in a 5% CO_2 incubator in MEM (alpha modification) containing 10% FCS and antibiotics (penicillin and streptomycin). They were checked regularly for mycoplasma contamination using the Gene-Probe kit. To minimise genetic drift, the cells were maintained in culture for no more than four to six weeks.

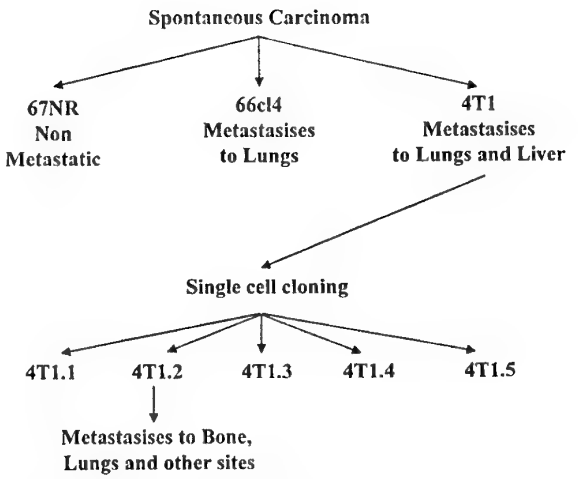


Figure 1. Flow chart showing the derivation of the breast tumour lines used in this study.

Prior to inoculation, cells were checked for viability using trypan blue.

In vivo *studies*

Female BALB/c mice aged 6–8 weeks were injected into the fourth mammary fat pad with 1×10^5 viable cells in a 10 μl volume, or into the lateral tail vein with 5×10^5 cells in a 0.2 ml volume, or into the left ventricle with 2×10^5 cells in a 0.2 ml volume.

Spontaneous metastasis from the mammary fat pad

Tumours were palpable 7–10 days after injection into the mammary fat pad. Primary tumour size was measured three times a week using callipers and the final tumour weight was measured after sacrificing the mice and excising tumours. Tumour burden in the lungs was quantitated by counting nodules after inflation of the lungs with India ink. Metastases in other organs were observed visually or by histological examination of formalin fixed samples. Blood was collected by cardiac puncture under anaesthetic at the time of sacrifice to obtain plasma for PTHrP and calcium assays. In some experiments, occult metastatic cells were quantitated by recovery of clonogenic cells from disaggregated tissues and growth in selective media as described previously [12]. By sacrificing groups of 5–6 animals at different times after initiation of the primary tumour, the kinetics of metastasis to the lung and bone were determined. Lungs were digested as previously described [12]. The spinal column was removed, individual vertebrae split open with scalpels, agitated, and stirred briefly in media containing 60 μM 6-thioguanine. The crushed spinal column was removed and the released cells were incubated for 7–10 days at 37 °C before colonies were fixed, stained, and counted.

Kinetics of bone colonisation following intravenous injections

Groups of 3–6 mice were sacrificed 7, 12, 17 and 19 days after intravenous injection of 5×10^5 tumour cells. Lungs were

enzymatically digested and vertebral columns were crushed and released cells plated in selective media as described above.

Bone metastasis following intracardiac injections

Tumour cells were injected into the left ventricle and hence directly into the arterial system. Mice were culled when they first showed signs of distress. The number of lung metastases was assessed by India ink inflation and the presence of tumour deposits in the bone was detected by examination of histological sections of the spine and femur.

Histology

Primary tumours, lungs, liver, femora and spines from tumour-bearing and control mice were fixed in 10% buffered formalin. Bones were decalcified in EDTA [13] and embedded in paraffin. Standard haematoxylin and eosin (H&E) staining procedures were employed for assessment of morphology. For detection of bone metastases, the paraffin embedded samples were cut into 5 μm sections and every twentieth section was stained and examined by microscopy.

Immunohistochemistry

PTHrP. PTHrP immunostaining was carried out as previously described [14] using a sheep polyclonal antibody raised against synthetic human PTHrP (50–69) at dilutions of 1/200 and 1/300 overnight at 4 °C. Normal sheep serum was used for non-immunecontrols and normal mouse skin was used as a positive control in all assays. Other controls included deletion of each of the reagents.

Keratin. A rabbit polyclonal anti-keratin (wide spectrum 2622 from Dako Corp.) was used overnight at dilutions of 1/500 and 1/750 at 4 °C. Antigen retrieval was achieved by microwave treatment in 0.05 M tris buffer pH10 and detection was by biotin-streptavidin-peroxidase.

Estrogen receptor. A monoclonal antibody against the human estrogen receptor (ER) (Novocastra NCL-ER-6F11), which cross-reacts with the mouse protein, was used overnight at dilutions of 1/250 and 1/500 at 4 °C. Antigen retrieval was achieved by microwave exposure in 0.01M citrate buffer, pH6 and detected with biotin-streptavidin-peroxidase.

In all cases, specific reaction is indicated by brown staining in the sections.

Radioimmunoassay for PTHrP

PTHrP was measured with an N-terminal directed antibody using a radioimmunoassay described previously [15]. To assay release of PTHrP from cultures, cells in an exponential phase of growth were incubated in serum-free medium containing 0.1% BSA for 1 h. The medium was then replaced with fresh serum-free medium containing 0.1% BSA for 20 h. PTHrP was measured on aliquots of the medium cleared of cell debris by centrifugation. To correct for cell numbers, the remaining monolayer of cells was rinsed with PBS, drained, dissolved in 1M sodium hydroxide overnight at 37 °C and the protein content measured using a modified Lowry assay.

Plasma calcium assay

Total calcium was measured using the Arsenazo III assay (Trace Scientific, Australia), following the recommended protocol.

Results

The kinetics and patterns of metastatic spread of three sublines, 67NR, 66cl4 and 4T1, derived from the original spontaneous carcinoma (Figure 1), were assessed in female BALB/c mice of 6–8 weeks of age following varying routes of inoculation. In addition, we have characterised a single cell clone of 4T1, called 4T1.2, that shows a strong propensity to metastasise to bone. In a previous analysis, 67NR was characterised as being non-metastatic, 66cl4 as metastasising to lung and 4T1 as metastasising to lung and liver [12].

Kinetics of 4T1 metastasis

After inoculation into mammary gland, clonogenic 4T1 cells were always recovered from the lungs prior to recovery from the bone. Clonogenic cells were recovered by day 15 (first day assessed) from the lungs but not from bone at a time when the median weight of the primary tumours was 216 mg. Clonogenic 4T1 cells were not recovered from bone at day 19 (6 mice tested) when the median weight of the primary tumours was 343 mg, but were recovered from vertebrae of 5 of 6 mice at day 22 when the median weight of the primary tumours was 546 mg. In similar experiments, clonogenic cells were not recovered from vertebrae of mice bearing 67NR or 66cl4 primary tumours. After intravenous injection, clonogenic 4T1 cells were recovered from lungs at all times. Lung metastases become grossly visible by day 19. Vertebrae were uniformly negative at days 7 and 12, but became uniformly positive by day 17 following injection. An attempt to isolate a clone of 4T1 for which clonogenic cells were not detected in the vertebrae was unsuccessful. Twenty clones were randomly isolated and injected intravenously. Although some clones appeared to be quantitatively more metastatic to bone than others, clonogenic cells were recovered from spines by day 20 in all cases. Subsequent analyses of overt tumour growth were performed using clone 2 of 4T1 (4T1.2).

Analysis of growth patterns of the breast carcinoma sublines

The pattern of metastatic spread of 67NR, 66cl4 and 4T1.2 was assessed following inoculation into the mammary gland. Mammary tumours were palpable within 7–10 days. The

tumours grew rapidly and the mice were killed when the tumours reached a weight of 1–2 g after 30–44 days. Some of the 4T1.2 tumour-bearing mice exhibited signs of partial paralysis of the hind limbs by this time. An analysis of primary tumour size, lung metastases and bone metastases is shown in Table 1, with data from two independent experiments (Experiments 1 and 2). The tumour lines exhibited different growth rates *in vivo* that did not mirror their growth rates *in vitro*. 67NR, which has a slower growth rate *in vitro*, grew more rapidly *in vivo* than the other two lines. 4T1.2, which has a similar growth rate *in vitro* to 66cl4, grew more slowly *in vivo* than 66cl4. The 4T1.2 line showed a greater capacity to metastasise to the lungs from the mammary gland compared to 66cl4, whilst almost no tumour nodules were detected in the lungs of mice inoculated with 67NR (one mouse was found to have one lung tumour nodule in Experiment 1). In other experiments, all mice were culled on the same day after tumour inoculation and despite 4T1.2 primary tumours being smaller, the mice bearing 4T1.2 tumours still exhibited more lung nodules (data not shown). From visual examination of the mice, it is evident that 66cl4 tumours do not colonize other organs apart from the lung, whilst mice bearing 4T1.2 tumours often had visible nodules on the diaphragm and rib cage and enlarged lymph nodes. By histological examination of the spine and femur, only the 4T1.2 clone exhibited tumour growth in the bone, confirming the assays for clonogenic tumour cells mentioned above.

Intracardiac injection of tumour cells

To investigate further whether the 67NR and 66cl4 cells are unable to reach bone or whether they can reach, but not grow in the bone environment, tumour cells were injected into the left ventricle and hence into the arterial system leading to bone. Mice were assessed 11–17 days later when they first showed signs of distress (Table 1, Experiment 3). Whilst all three tumour lines showed an ability to colonise the lung, no histological evidence of tumour growth was found in the spine or femurs of four mice examined after inoculation of 66cl4 tumour cells. One of four mice examined had 67NR tumour growing in bone while two out of three mice carrying 4T1.2 tumour cells had tumour masses in the bones (Table 1).

Histological analysis of primary and secondary tumours

Primary tumours that were 10–15 mm in diameter showed areas of necrosis towards the central regions. All comprised sheets and cords of large undifferentiated cells typical of carcinoma. Large numbers of mitotic figures were apparent. There were no obvious differences in morphology of tumours generated from the different cell lines (see Figure 2A for the 4T1.2 primary tumour). Pleural, subpleural and perivascular tumour nodules were evident in the lungs of mice bearing 66cl4 and 4T1.2 tumours (not shown). No tumour masses were detected in the livers of any animals although extramedullary hemopoiesis developed in livers of 4T1.2 tumour-bearing mice (not shown).

In the femora of 4T1.2 tumour-bearing mice, a single metastasis generally developed in the distal diaphysis, close to the growth plate and in some cases occupied large areas of the marrow space by 5 weeks post tumour inoculation (Figures 2E, F). Occasionally, attached muscle adjacent to the bone metastasis became involved. Tumours grew in the spines of 4T1.2 animals in multiple vertebrae and often involved surrounding muscle (Figure 2G). Tumour growth in the spine led to complete replacement of bone marrow by tumour cells and invasion progressed into the spinal canal. Areas of active osteolysis could be seen adjacent to trabecular bone surfaces in both the spine (Figure 2H) and femur (not shown) of 4T1.2 bearing animals.

PTHrP, keratin and ER expression in primary and secondary tumours

Primary tumours generated by all three cell lines stained positively for PTHrP (see Figure 2C for 4T1.2). In tumours from 66cl4 and 4T1.2 cells, staining was usually most intense in dividing cells with mitotic figures, and at the edges of the tumours where normal tissue invasion was occurring. Tumours from 67NR cells tended to stain more weakly, with mitotic cells weakly positive or negative for PTHrP (not shown). Metastases in lungs also stained weakly positive for PTHrP. In both the femur and spine, 4T1.2 tumours were positive for PTHrP but there was no apparent difference in intensity from that seen in the tumour at the primary site (Figures 2C and E). For comparison, the PTHrP staining of a 4T1.2 tumour in femur and a non-immune control are shown (Figures 2E and F, respectively). Strong staining for keratin and weaker staining for the ER was evident for all three primary tumour types (see Figure 2B and D for keratin and ER immunostaining in 4T1.2 tumours, respectively). 4T1.2 tumours in bone also stained positively for keratin (not shown).

PTHrP secretion

PTHrP levels were measured both in the medium of cultured cells and in the plasma of tumour-bearing mice. PTHrP secretion into the medium of the tumour sublines grown in culture was measured in exponentially growing cells placed in serum-free medium for 20 h. As shown in Figure 3, PTHrP levels were highest in the 4T1.2 line, intermediate in 66cl4 cells and negligible in 67NR cells. Plasma PTHrP levels in tumour-bearing mice are shown in Table 2. Compared to the value in non-tumour-bearing mice, plasma PTHrP was elevated approximately 1.7 fold in mice bearing 4T1.2 tumours. This increase was significant ($P < 0.04$) when analysed using a Student t test.

Plasma calcium levels

Circulating calcium levels are presented in Table 2 along with the PTHrP data. In parallel with the elevation of plasma PTHrP levels in mice bearing 4T1.2 tumours, there was a significant increase in plasma calcium. In mice bearing 67NR tumours, calcium levels were also significantly elevated but to a lesser degree. No hypercalcaemia was evident in mice bearing 66cl4 tumours.

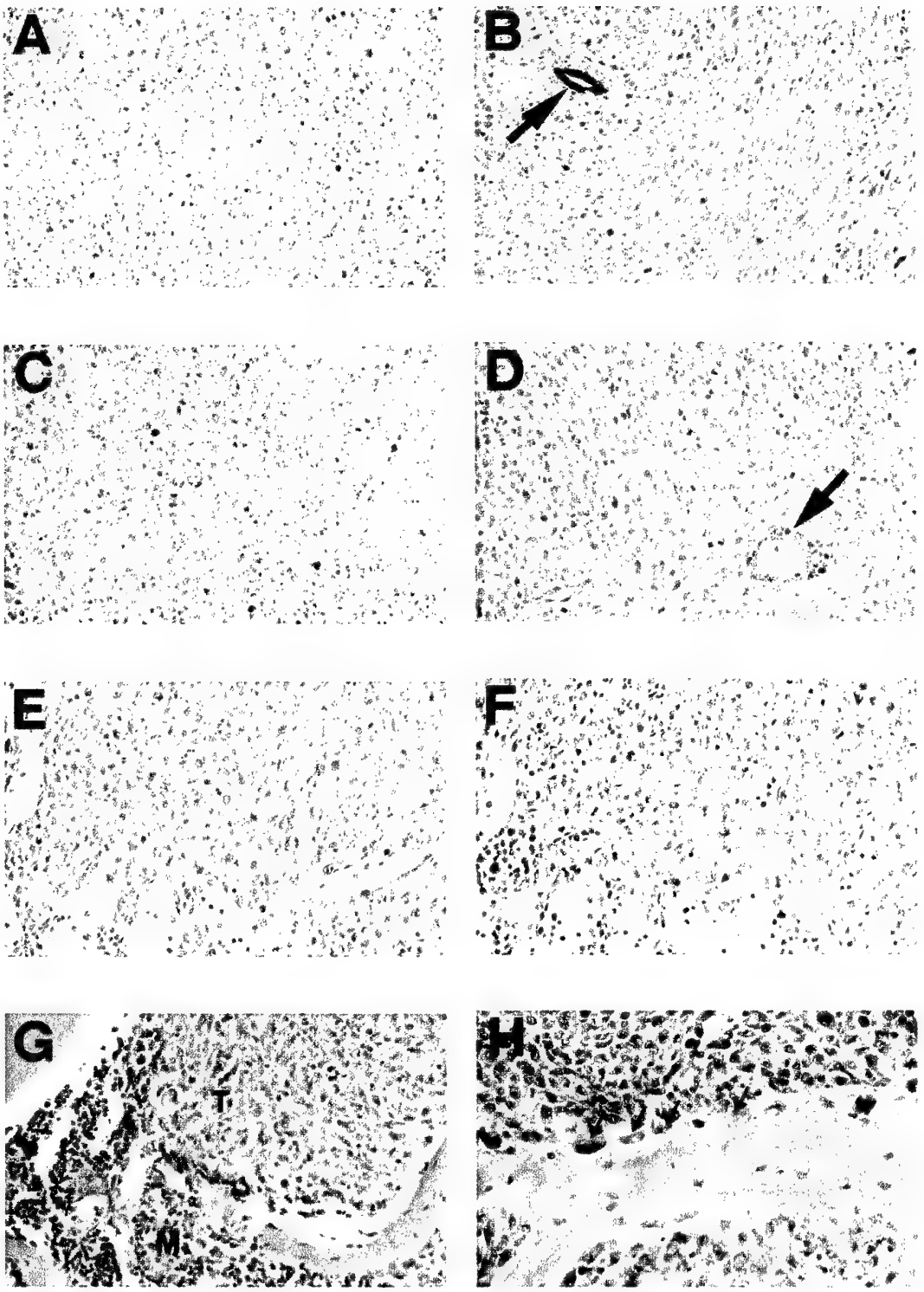


Figure 2. Morphological and immunohistochemical analysis of the 4T1.2 tumour. Section of the primary tumour showing morphological appearance by H&E staining (A) and immunostaining of the primary tumour for keratin (B), PTHrP (C) and estrogen receptor (D). Brown staining indicates regions of specific immunostaining. The arrows in **B** and **D** indicate strong immunostaining in epithelial cells lining the ducts. PTHrP immunostaining of a section through the femur, showing the growth plate and PTHrP positive tumour cells adjacent to it (E). Non-immune control of adjacent section of the femur to E (F). An H&E stained section through the spine showing a region of tumour (T) and a region of normal marrow (M) (G). **A–G** were photographed at ×80 magnification to show distribution of specific proteins throughout the tumour. A higher power image (×100) of a section of the spine stained with H&E showing the large multinucleated osteoclasts (indicated with arrows) adjacent to the trabecular bone, causing osteolysis of the bone (H).

Table 1. Tumour growth characteristics after injection of cells into the mammary fat pad or into the left ventricle.

Tumour subline	No. Mice	Time of growth (days, mean±SE)	Tumour weight (g) (mean±SE)	Lung metastases (mean±SE)	Bone metastases (No.+ve mice) Femur	Spine
Experiment 1						
67NR	7	32.6±0.4	2.3±0.8	0.2±0.4	0/3	0/3
66cl4	6	32.8±0.4	1.3±0.2	2.3±2.6	0/3	0/3
4T1.2	6	34.0±0	0.9±0.3	22.5±8.5	3/3	1/3
Experiment 2						
67NR	8	44.1±1.8	2.1±0.2	0	0/4	0/4
66cl4	14	30.6±1.4	1.2±0.1	15.7±1.6	0/4	0/4
4T1.2	15	36.6±0.9	1.0±0.1	34.3±2.7	4/4	3/4
Experiment 3						
67NR	5	17.0±0	–	12.2±6.0	0/4	1/4
66cl4	5	11.0±0	–	213.0±64.1	0/4	0/4
4T1.2	5	14.8±0.2	–	82.4±16.0	0/3	2/3

For the spontaneous metastasis assays shown in Experiments 1 and 2, 1×10^5 tumour cells were inoculated into the fourth mammary fat pad. Mice were culled when the primary tumour reached a size of approximately 1g or when the mouse first showed signs of distress. For the intra-cardiac experiment shown in Experiment 3, 2×10^5 tumour cells were inoculated into the left ventricle of the heart and mice were culled when they first showed signs of distress. Where appropriate, the primary tumour was excised and weighed. Lung metastases were scored after India ink inflation of the lungs. Tumour deposits in bone were detected by examination of H&E sections of the spine and femur.

Table 2. Plasma concentrations of PTHrP and calcium in mice bearing 67NR, 66cl4 and 4T1.2 tumours.

Cell Line	Plasma PTHrP (pM) Mean ± SE	Plasma Ca (mM) Mean ± SE
67NR	8.7±0.7 ($n = 15$)	2.33±0.03* ($n = 15$)
66cl4	4.7±0.5 ($n = 21$)	2.24±0.05 ($n = 20$)
4T1.2	12.1±1.5* ($n = 19$)	2.37±0.04** ($n = 13$)
Control mice	7.6±1 ($n = 13$)	2.21±0.02 ($n = 13$)

The samples were obtained from plasma taken when the mice were killed at the end of the experiment (Day 32–36 post inoculation). The 'n' values indicate the number of mice analysed in each group. The P values were calculated using a two sample Student t test assuming unequal variances. Data with a single asterisk showed a P value < 0.05 whilst those with a double asterisk had a P value <0.005 compared to values obtained from control mice. The other data were not significantly different to values obtained in control mice. The controls were age matched, non-tumour bearing mice.

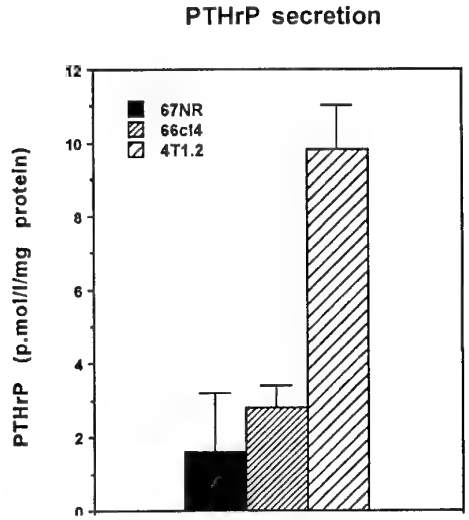


Figure 3. PTHrP secretion by the tumour sublines. PTHrP in the medium of cells in exponential phase of growth was measured by RIA and standardised to the protein content of the cells in the sample.

Discussion

Although much has been learned about the process of metastasis, less is understood of the mechanisms that dictate why tumours spread successfully to one particular site and not another. The establishment of a tumour in a tissue depends both on factors that it expresses and those released locally [16]. The concept that the microenvironment of an organ provides the fertile 'soil' for growth of a tumour cell was first proposed by Paget over one hundred years ago [17]. Bone is the favoured site of metastases of breast, prostate and lung cancers and thus the bone microenvironment must provide an appropriate fertile 'soil' for these tumours [16]. Once present in bone, tumour cells cause disruption of normal bone remodeling, with promotion of osteoclast formation and enhanced osteolysis usually occurring in the case of breast tumours. We have described here a clinically relevant model of breast cancer metastasis in which primary tumour cells spread from the mammary gland to bone and other distant sites. This model, with its use of subpopulations of cells that metastasise from the primary site in the mammary

gland to different organs, now provides the means to address the mechanisms of site-specific metastasis in a clinically relevant setting.

The 4T1.2 clonal cell line that we describe here metastasises to lung and bone following orthotopic inoculation into the mammary fat pad. The parental line 4T1 was shown previously to spread to lungs and liver [12] and here we show that clonogenic cells can be detected in spines of 4T1 bearing mice. Kinetic studies revealed that clonogenic cells could be detected in the lung prior to detection in the spine. As shown previously, the sister populations, 66cl4 and 67NR metastasised to the lung (66cl4) or did not metastasise at all (67NR). 4T1.2 cells generated tumours in both femur and spine and these were accompanied by local destruction of bone with active osteoclasts visible along bone surfaces. In none of our experiments with 4T1.2 did we observe tumours in the liver, although extramedullary hemopoiesis was observed.

Intracardiac inoculation confirmed that only the 4T1.2 cells were consistently metastatic to bone. This route of inoculation also led to an increased incidence of lung metastases in all three groups, including the normally non-metastatic 67NR tumours. No bone metastases were seen in mice inoculated with 66cl4 cells but spine involvement was observed in one of three mice inoculated with 67NR cells. This suggests that the 67NR cells may have some ability to grow in bone but are deficient in the mechanism required to detach from the primary site.

Our preliminary characterisation of this model has included use of keratin immunohistochemical staining to confirm the epithelial nature of the tumour cells and identification of ER in primary tumours generated from all three cell lines. Keratin staining was evident in all tumour sublines and was used as a guide to the identification of tumour cells at metastatic sites. The presence of the ER in the primary tumours, as evidenced by immunological staining, indicates an opportunity for the evaluation of anti-estrogen therapies in the modulation of site specific metastatic spread.

Since there is strong evidence implicating PTHrP in the metastatic spread of breast cancer to bone [18], we examined whether its expression correlated with bone metastasis in this model. PTHrP is the major mediator of hypercalcaemia in cancer patients including those with lung and breast cancers [19]. The primary tumours of all three cell lines investigated here stained positively for PTHrP, as did the bone metastases of 4T1.2 tumours. There were no major differences in the intensity of staining of this protein in the tumours generated from each of the cell lines indicating that, at the primary site, the presence of PTHrP in the tumour alone is insufficient to direct spread and growth specifically in bone. Nevertheless, levels of PTHrP secreted by the 4T1.2 cells *in vitro* were higher than those secreted by the 66cl4 or 67NR. Thus, it would seem that the higher level of cellular PTHrP secretion *in vitro* may be associated with the ability to generate bone metastases *in vivo*. Furthermore, levels of circulating PTHrP were raised significantly in mice bearing 4T1.2 tumours relative to controls and were higher than in 67NR and 66cl4 tumour-bearing animals. This may reflect increased release of PTHrP from tumour both at the primary site and in bone. Consistent with this and the known activity of PTHrP on bone was the observation that circulating calcium was slightly raised in animals inoculated with 4T1.2 cells.

The increased calcium levels observed in the 67NR tumour bearing mice, in which PTHrP levels were not raised, may reflect an alternative mechanism of osteolysis that could have facilitated tumour growth in the spine of one mouse inoculated via the intracardiac route with 67NR cells. Assessment of the expression and regulation of other bone resorbing cytokines in these cells will be of interest.

Whilst evidence for the role of PTHrP in the establishment of breast tumours in bone is convincing, it remains to be established whether other bone resorbing factors may be produced by the tumours to provide alternative or complementary mechanisms. Manipulation of the level of PTHrP expression by transfection or by clonal selection of the 4T1, 66cl4 and 67NR sublines will aid in addressing the importance of PTHrP secretion in directing site specific spread from the primary tumour, and its ability to be regulated in bone. We have derived clones of 4T1 with varying levels of PTHrP secretion and clones in which no PTHrP secretion can be detected. In experiments underway, the dependence on PTHrP expression for metastasis to bone and other sites is being tested using these clones. By analysing and comparing the gene expression profiles of the different sublines, we will be able to determine the contribution of other factors to the processes of homing and growth in bone. While this approach may endorse a role for PTHrP in promoting bone metastasis, it is likely to show that additional factors also have a critical function in homing to bone and in the development of bone metastases. In particular, it is likely that other bone resorbing cytokines, such as interleukin 6 [3] and interleukin 11 [4] may be as effective as PTHrP in enhancing bone metastasis formation.

This is the first natural model of mammary metastasis to bone to be reported. The model provides an excellent experimental system in which to investigate the factors that control metastatic spread of breast cancer to specific sites, particularly bone, using genetically matched clones that do or do not metastasise to bone. An advantage of this unique system is that it uses subpopulations of a spontaneous metastatic tumour and represents the entire metastatic process from the primary site. The model is ideal for definition of the molecular mechanisms of site-specific metastasis and for the evaluation of therapeutic strategies such as anti-estrogens, and thus will contribute significantly to the clinical diagnosis and management of breast cancer patients.

Acknowledgements

This project was sponsored in part by the US Department of the Army, Grant Number DAMD17-98-1-8144, in part by a Program Grant from the National Health & Medical Research Council of Australia and in part by the Chugai Pharmaceutical Company of Japan. The content of the information does not necessarily reflect the position or the policy

of either government and no official endorsement should be inferred.

References

1. Mundy GR. Mechanisms of bone metastasis. Cancer 1997; 80: 1546–56.
2. Mundy GR, Yoneda T. Mechanisms of bone metastasis. In: Orr FW, Singh G (eds) Bone Metastasis – Mechanisms and Pathophysiology: New York: Springer-Verlag 1996; 1–16.
3. Crichton MB, Nichols JE, Zhao Y, Bulun SE, Simpson ER. Expression of transcripts of interleukin-6 and related cytokines by human breast tumors, breast cancer cells, and adipose stromal cells. Mol Cell Endocrinol 1996; 118: 215–20.
4. Lacroix M, Siwek B, Marie PJ, Body JJ. Production and regulation of interleukin 11 by breast cancer cells. Cancer Lett 1998; 127: 29–35.
5. Oates AJ, Barraclough R, Rudland PS. The identification of osteopontin as a metastasis-related gene product in a rodent mammary tumour model. Oncogene 1996; 13: 97–104.
6. Gillespie MT, Thomas RJ, Pu ZY, Zhou H, Martin TJ, Findlay DM. Calcitonin receptors, bone sialoprotein and osteopontin are expressed in primary breast cancers. Int J Cancer 1997; 73: 812–15.
7. Guise TA. Parathyroid hormone-related protein and bone metastases. Cancer 1997; 80: 1572–80.
8. Price JE. Metastasis from human breast cancer cell lines. Breast Canc Res Treat 1996; 39: 93–102.
9. Kjønniksen I, Winderen M, Bruland Ø, Fodstat Ø. Validity and usefulness of human tumor models established by intratibial cell inoculation in nude rats. Cancer Res 1994; 54: 1715–19.
10. Arguello F, Baggs RB, Eskenazi AE, Duerst RE, Frantz CN. Vascular anatomy and organ-specific tumor growth as critical factors in the development of metastases and their distribution among organs. Int J Cancer 1991; 48: 583–90.
11. Miller BE, Roi LD, Howard LM, Miller FR. Quantitative selectivity of contact-mediated intercellular communication in a metastatic mouse mammary tumor line. Cancer Res 1983; 43: 4102–7.
12. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumour. Cancer Res 1992; 52: 1399–405.
13. Kartsogiannis V, Moseley JM, McKelvie B, Chou ST, Hards DK, Ng KW, Martin, TJ, Zhou, H. Temporal expression of PTHrP during endochondral bone formation in mouse and intramembranous bone formation in an in vivo rabbit model. Bone 1997; 21: 385–92.
14. MacIsaac RJ, Caple IW, Danks JA, Diefenbach-Jagger H, Grill V, Moseley JM, Southby, J, Martin, TJ. Ontogeny of parathyroid hormone-related protein in the ovine parathyroid gland. Endocrinology 1991; 129: 757–64.
15. Grill V, Ho P, Body JJ, Johanson, N, Lee SC, Kukreja SC, Moseley, JM, Martin, TJ. Parathyroid hormone-related protein: elevated levels both in humoral hypercalcemia of malignancy and in hypercalcemia complicating metastatic breast disease. J Clin Endocrinol Metabol 1992; 73: 110–15.
16. Meyer T, Hart IR. Mechanisms of tumour metastasis. Eur J Cancer 1998; 34: 214–21.
17. Paget S. The distribution of secondary growths in cancer of the breast. Lancet 1889; 1: 571–73.
18. Guise TA, Yin JJ, Taylor SD, Kumagai Y, Dallas M, Boyce BF, Yoneda, T, Mundy, GR. Evidence for a causal role of parathyroid hormone-related protein in the pathogenesis of human breast cancer-mediated osteolysis. J Clin Invest 1996; 98: 1544–49.
19. Rankin W, Grill V, Martin TJ. Parathyroid hormone-related protein and hypercalcemia. Cancer 1997; 80: 1564–71.

Clinical & Experimental Metastasis 0: 1–8, 2001.

MMP-9 secretion and MMP-2 activation distinguish invasive and metastatic sublines of a mouse mammary carcinoma system showing epithelial-mesenchymal transition traits

FIRST PROOF
AUTHOR'S PROOF

Angus M. Tester[1,4], Neeracha Ruangpanit[1], Robin L. Anderson[3] and Erik W. Thompson[1,2]
[1]*VBCRC Breast Cancer Invasion and Metastasis Unit, St. Vincent's Institute of Medical Research;* [2]*University of Melbourne Department of Surgery, St. Vincent's Hospital, and* [3]*Peter MacCallum Cancer Institute, Melbourne, Australia;* [4]*Current address: Department of Oral Biological and Clinical Sciences, University of British Columbia, Vancouver, British Columbia, Canada*

Received 5 April 2001; accepted 23 May 2001

Key words: EMT, epithelio-mesenchymal transition, mammary, metastasis, MMP-2 activation, MT1-MMP, murine, TIMP-2, vimentin

Abstract

We have investigated the gelatinase profiles and invasiveness of clonal tumour sublines derived from a spontaneously arising mammary tumour in a Balb/cfC3H mouse. The 67NR, 66cl4 and 4T1.2 sublines have low, intermediate and high metastatic potential respectively. In Boyden chamber studies, Matrigel invasion was seen to be progressively higher in the more metastatic lines 4T1.2 > 66cl4 > 67NR, consistent with MMP-2 activation potential, MMP-9 secretion, and migration over either type I or IV collagen, which were low in both 67NR and 66cl4 cells compared to 4T1.2 cells. These attributes are consistent with those seen in human breast cancer cell lines which appear to have undergone an epithelial-mesenchymal transition (EMT) as indicated by vimentin expression. We were, however, surprised to find vimentin expression, MT1-MMP expression and stellate Matrigel outgrowth in the non-invasive, non-metastatic 67NR cells, indicating that they had undergone an EMT despite not being invasive. We conclude that the EMT is manifested to differing degrees in these three clonal cell lines, and that the 67NR cells have either undergone a partial EMT or have since lost certain important attributes of the EMT-derived phenotype. This model should prove useful in further characterizing the regulation of MT1-MMP mediated MMP-2 activation and delineating the EMT in breast cancer progression.

Introduction

For cancer dissemination, metastatic cells must escape from the primary tumour and then invade and migrate to a secondary site. This process requires the proteolytic degradation of extracellular matrix components such as basement membranes and interstitial matrices that encapsulate the tumour and secondary organ/site. Several classes of proteinases have been implicated in these processes; these include matrix metalloproteinases (MMPs), serine proteinases and cysteine proteinases [1–3]. MMP-2 and -9, which comprise the gelatinase MMP sub-family, are believed to play a critical role in tumour invasiveness. These enzymes are able to degrade collagen type IV, which is a major structural component of basement membranes [4] and thus may enable the escape of cancer cells. The gelatinases also efficiently degrade denatured collagen, and are abundant at many sites of tissue remodeling. These enzymes are largely produced by the reactive stromal cells around breast tumours [5–8], but also are produced by breast cancer cell lines which have adopted mesenchymal traits indicative of an epithelial to mesenchymal transition (EMT; [9]).

After lymph node, bone is the second most common organ for breast cancer metastasis, and metastatic cells have been detected in up to 80% of patients who die from the disease [10]. The 4T1 murine mammary tumour model was recently adapted to investigate metastasis from the mammary gland to bone [11, 12]. This model is among the first to show spontaneous metastasis of breast cancer cells from the primary site to bone, and mimics closely the pattern of metastatic spread observed in human breast cancer. This provides a more complete representation than existing models that require the cancer cells to be injected into the arterial system via the left ventricle [10, 13] or directly into the tibia [14] in order to induce bone metastasis. Derived originally from a spontaneously arising mammary tumour in a Balb/cfC3H mouse [11] the clonal lines show varying metastatic potential; non-metastatic (67NR), those that metastasise only to lung (66cl4), or to liver in addition to lung (4T1). The bone-metastatic 4T1.2 subline, which was derived by single cell cloning of the 4T1 line, repro-

Correspondence to: Erik W. Thompson, VBCRC Invasion & Metastasis Unit, St. Vincent's Institute of Medical Research, 41 Victoria Pde., Fitzroy, 3065, Australia. Phone +61-3 9288-2480; Fax +61-3-9416-2676, E-mail: rik@medstv.unimelb.edu.au

ducibly forms metastases in numerous sites in addition to bone following implantation into the mammary fat pad [12].

This work compares gelatinase profiles and the *in vitro* invasive potential of the cell lines with their *in vivo* metastatic potential in the mouse.

Materials and methods

Cell Culture

The 67NR, 66cl4, and 4T1 cell lines were first derived and kindly supplied by Dr F. Miller (Karmanos Cancer Centre, Detroit, Michigan) [11]. Further cloning of the 4T1 cells produced the 4T1.2 subline which shows spontaneous metastasis to bone from the mammary fat pad [12]. Cell lines were cultured in αMEM (Gibco BRL; Gaithersburg, Maryland) supplemented with 10% fetal bovine serum (FBS; Gibco BRL; Gaithersburg, Maryland) and antibiotics (gentamycin and minocycline; Sigma, St Louis, MD). Cells were checked regularly for mycoplasma contamination using the Gen-Probe kit (Gen Probe; San Diego, California).

Invasion assay

Cells (5.6×10^4) were suspended in medium (56 μl) containing 0.1% (w/v) BSA and placed in the upper compartment of a 48 well invasion chamber (Neuroprobe; Cabin John, Maryland). They were separated from the chemoattractant (28 μl αMEM/10% FBS) in the lower compartment by a porous filter (polycarbonate, 8-μm pore size; Osmonics; Livermore, California) coated with Matrigel (20 μg/cm^2/Becton Dickinson; Bedford, Maryland). After incubation at 37 °C for 6 h the filters were fixed in methanol and stained with eosin and hematoxylin (Quick Dip 1 and 2; Histolabs, Fronine, Sydney, Australia). The cells on the upper surface of the filter were wiped away with a cotton swab. Cells on the lower surface of the filter were counted in 4 different fields at 20× magnification. Each cell line was assayed 4 times and each assay performed in triplicate. Statistical analysis was performed by one way ANOVA.

Migration assay

Migration assays were performed as described for the invasion assay, but the filters were incubated with the various substrates overnight at 10 μg/ml for collagen type I and Matrigel and at 40 μg/ml for collagen type IV. The assay was incubated for 5 h at 37 °C. Background migration towards αMEM/0.1% BSA was subtracted from all results. Each cell line was assayed 2–4 times on each substrate and assays were performed in triplicate. Statistical analysis was performed by one way ANOVA.

Matrigel outgrowth assay

Matrigel outgrowth assays were performed in 48 well plates. The cells (2×10^4) were dispersed in 75 μl of undiluted Matrigel (10 mg/ml) and then overlaid onto 100 μl of polymerized Matrigel. Once the top layer had polymerized the cells were cultured in αMEM/10%FBS for up to 10 days and photographed at 20× magnification.

Analysis of MMP-2 activation

Cells were plated (5×10^4 cells/0.5 ml αMEM/FBS) in a 24-well plate, either directly onto the plastic or on top of a polymerized gel of collagen type I (0.5 ml @2 mg/ml; Vitrogen, Palo Alto, California). The cells were allowed to adhere overnight, then washed in unsupplemented media twice before incubation in either serum free medium (SFM; αMEM containing 1× ITS, 1× non essential amino acids, 1 mM sodium pyruvate, 1× vitamins and 0.1% BSA; all from Sigma; St. Louis, Missouri) or SFM supplemented with exogenous pro-MMP-2 as described previously [16]. Concanavalin A (Con A; 20 μg/ml) was included in some cultures to induce activation of MMP-2 as described previously [16]. Cultures were maintained up to 96 h on the collagen gel and 48 h with Con A, with appropriate time point controls cultured on plastic. Aliquots of the conditioned media were removed every 24 h following treatment and stored at −20 °C until testing for the activation of the exogenous pro-MMP-2 by gelatin zymography.

Gelatin zymography analysis

Conditioned media samples were subjected to SDS-PAGE on 8% gels copolymerized with 0.15% gelatin (w/v). The gels were washed in 2.5% Triton X-100 for an hour and then incubated overnight at 37 °C in 10 mM $CaCl_2$, 50 mM Tris HCl, 0.15 M NaCl, 1% Triton X-100, and 0.025% sodium azide. Bands were visualized by staining with Coomassie R-250.

Western analysis of MT1-MMP, vimentin and TIMP-2 protein levels

Cells were plated (1×10^5) in 6 well plates, either on top of a 1.0 ml gel of collagen type I (2 mg/ml) or onto the culture plastic. At various time points the cells were removed from the collagen gel with collagenase type II (~200 units; Worthington Biochem. Corp., New Jersey) and scraped into a modified RIPA buffer (50 mM Tris/HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 10 μg/ml Aprotinin, 10 μg/ml Leupeptin, 1 mM sodium orthovanadate, 1 mM sodium fluoride). The cells were lysed and the protein content determined by the BCA protein assay (Pierce, Rockford, Illinois) according to manufacture's instructions. Samples (25 μg of protein) were subjected to SDS-PAGE on 8% mini gels and transblotted onto PVDF membrane (Millipore). The membrane was blocked in 5% skim milk, 0.05% Tween in PBS for 2 h at room temperature before exposure to the primary antibody overnight at 4 °C [mouse monoclonal anti-MT1 antibody (1 μg/ml; clone 114-1F2; Oncogene Research Products, Massachusetts) [17], goat polyclonal anti-vimentin (0.2 μg/ml; clone C-20; Santa Cruz; Santa Cruz, California), or mouse monoclonal anti-TIMP-2 (1 μg/ml; clone 67-4H11, kindly provided by Dr Kazushi Iwata, Fuji Chemical Co, Japan)]. The membranes were then incubated with

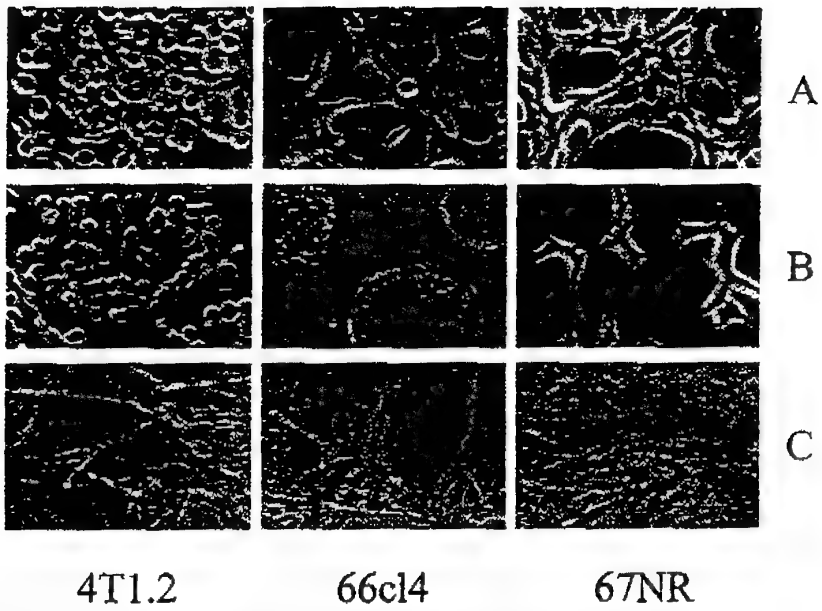


Figure 1. Cell Morphology. The cell lines were cultured on plastic (A) on a gel of fibrillar collagen I (2 mg/ml) for 72 h (B), or within Matrigel (10 mg/ml) for 5 days (C). Images of the cell morphology were captured from a phase microscope at 20× magnification.

contrast *

secondary horseradish-conjugated goat anti-mouse or rabbit anti-goat antibody (diluted 1/20,000; Pierce, Rockford, Illinois). Signals were developed with the SuperSignal kit (Pierce, Rockford, Illinois).

Results

Cell morphology

Distinct morphologies were adopted by the three cell lines when cultured on plastic or on a 3 dimensional collagen gel (Figure 1). The metastatic cell line 4T1.2 appeared rounded on plastic or collagen compared to the 66cl4 and 67NR cells, which adopted a stellate, flatter morphology and showed greater cell-cell contact on both substrates. Cell-cell contact was especially evident when 66cl4 cells were cultured on collagen, which resulted in the cells growing as spheroids. The morphology of the cells grown within Matrigel was observed over a 10 day period. The non metastatic 67NR cell line was the fastest growing, and formed a more extensive stellate outgrowth compared to the slower growing 66cl4 and 4T1.2 cells (Figure 1). The latter two lines also formed a similar stellate morphology but required 3–4 days before outgrowth was observed

Vimentin expression

The expression of vimentin at the protein level was analyzed by Western blot using the anti-vimentin antibody (Figure 2). There was no significant difference between the non-metastatic 67NR, and metastatic 4T1.2 cell levels of production. Interestingly, the intermediate line 66cl4 produced lower levels of vimentin. This was confirmed by immunofluorescence labeling of the cultured cells on glass

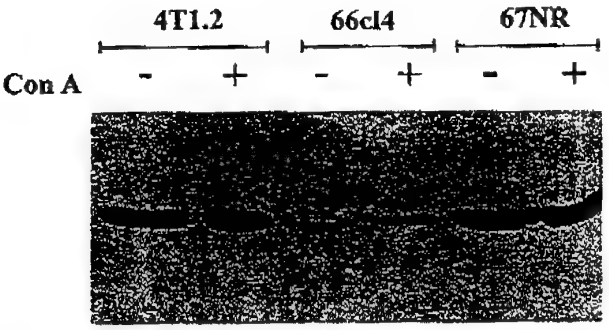


Figure 2. Vimentin Expression. Cells were cultured on plastic in the presence or absence of Con A (20 μg/ml) for 24 h. Whole cell lysates were prepared and analyzed by Western blot using an anti-vimentin antibody (C-20). Gel loading was standardized for equal protein loading.

(hyphen)

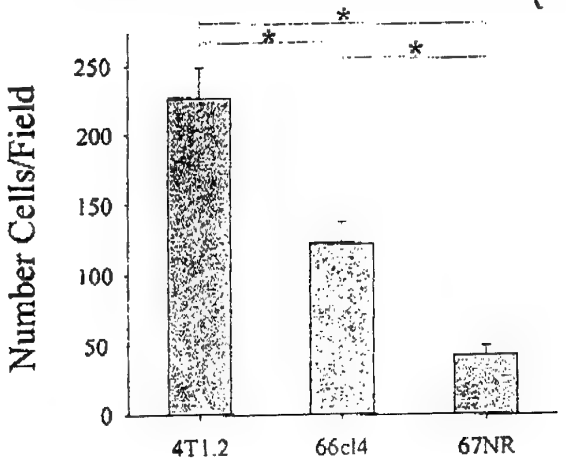


Figure 3. Invasive Potential of the Cells. The ability of the cells to invade Matrigel (20 μg/cm^2) was analyzed by chemo-invasion in microchemotaxis chambers. The assay was performed for 6 h. The bars represent the mean ±SEM (n = 9). Statistical analysis was performed by one way ANOVA and * indicates a P value <0.001.

slides. All three cell lines showed the incorporation of vimentin into an elaborate meshwork of intermediate filaments (data not shown). The production of vimentin was not regulated by collagen type I (data not shown) or Con A (Figure 2) for any of the cells. There was an occasional indication of a slight up-regulation of vimentin expression observed in the 66cl4 cells with Con A, but this was not a consistent finding.

Invasion and migration assays

The ability of the cell lines to penetrate and transgress a Matrigel barrier was investigated. The 4T1.2 cell line exhibited significantly ($p < 0.001$) greater invasive potential than the 66cl4 cell line, which in turn was significantly ($p < 0.001$) more invasive than the 67NR cell line (Figure 3).

The motility of the cell lines was tested on Matrigel, collagen type I and collagen type IV (Figure 4). The 4T1.2 cell line was significantly ($p < 0.001$) more motile than the 66cl4 and 67NR lines on all the substrates analyzed.

Production of gelatinases

Since the invasiveness of cell lines often correlates with gelatinase expression [18], the production of gelatinases was analyzed in 96 hour SFM supernatants. 4T1.2 cells showed higher production of MMP-9 compared to 66cl4 and 67NR in either the absence (Figure 5) or presence (Figure 6A) of collagen. None of the lines produced detectable levels of MMP-2.

Activation of exogenous MMP-2

MMP-2 activation is a hallmark of invasive human breast cancer cell lines [19]. We looked at the ability of the cell lines to activate exogenous MMP-2 in the presence or absence of collagen type I or Con A. The gelatin zymogram (Figure 6B; - lanes) shows that none of the cell lines were able to constitutively activate the pro-MMP-2 when cultured on plastic alone for 48 h (no activation was observed up to 96 h, not shown). The 4T1.2 cells were induced to activate latent MMP-2 by either collagen type I or Con A, as evidenced by the generation of lower molecular weight species that represent the intermediate and fully active forms of MMP-2. The active species were observed following treatment with either collagen or Con A for 24 h, and their levels increased over time. The 66cl4 cell line was induced to activate MMP-2 by collagen type I to a lesser extent than 4T1.2. Lower levels of the active species of MMP-2 were observed following 72 h of treatment with collagen, and Con A did not noticeably induce activation. There was no evidence for MMP-2 activation by the non-metastatic cell line 67NR over the 96-h period after treatment with either agent. The different response of the cell lines to collagen induced MMP-2 activation is not due to differences in the cells ability to adhere to the substrate (data not shown).

Expression of MT1-MMP and TIMP-2

Since both MT1-MMP and TIMP 2 are involved in the cell surface activation of MMP-2 [20], their levels were analyzed. Western blot (Figure 7A) indicates that MT1-MMP is produced by all three cell lines at similar levels. The MT1-MMP band co-migrates with the 60 kDa mature form of MT1-MMP observed in the human breast cell line MDA-MB-231 [16, 19]. Also Con A increased the amount of MT1-MMP observed in the cell lysates from all three cell lines with no discernable difference in the induction of MT1-MMP by Con A between the lines. Although MT1-MMP expression in the 4T1.2 cells is elevated compared to the other cells, the 67NR cells still express MT1-MMP at a level that would be sufficient for human breast cancer cells to activate pro-MMP-2. TIMP 2 expression was also analyzed and the results (Figure 7B) show that there was no obvious difference in the protein level of TIMP-2 present either in the cell lysates or released into the culture medium from the three cell lines. ~~Although~~ there is a slight decrease in the level of TIMP-2 in the 66cl4 cell lysate. Thus, MT1-MMP and TIMP-2 do not explain the differences observed in MMP-2 activation.

If possible: delete "Although" (not a sentence)

Discussion

The mouse model developed by Lelekakis et al. [12], utilizes three sister sublines that have different metastatic potentials following orthotopic implantation into the mammary fat pad; a highly metastatic line that consistently forms bone and lung metastases (4T1.2), an intermediate line with less metastases to the lung (66cl4), and a cell line which does not metastasize (67NR). The 4T1.2 line closely represents the profile of metastatic spread that is observed in human disease, and provides a basis for the *in vitro* analysis of characteristics related to metastatic progression.

Morphological analysis of the mammary carcinoma cell lines showed that the less metastatic 66cl4 and 67NR cell lines were flatter and had greater cell-cell contact compared to the metastatic 4T1.2 cell line, especially when cultured on collagen. When the cells were grown within Matrigel the 67NR cells were the fastest growing and formed more stellate outgrowth than the 66cl4 or 4T1.2 cells. We have previously associated lack of contact and stellate morphology with invasiveness and vimentin expression in human breast cancer cell lines [21, 22], and this has also been documented in other systems (reviewed in [9]). The expression of vimentin (an intermediate filament protein) and lack of cell-cell contact is thought to reflect epithelial to mesenchymal transition (EMT) – like changes similar to those seen in development [23]. Interestingly, when the vimentin expression by the mouse cell lines was investigated by Western blot, the non-invasive cell line 67NR was found to produce similar high levels to the 4T1.2 cells, compared to the 66cl4. The expression of vimentin in all three cell lines, and the high level seen in the 67NR, suggests that the 67NR cells exhibit some aspects of a mesenchymal phenotype. Indeed the 67NR morphology resembles many of the invasive human

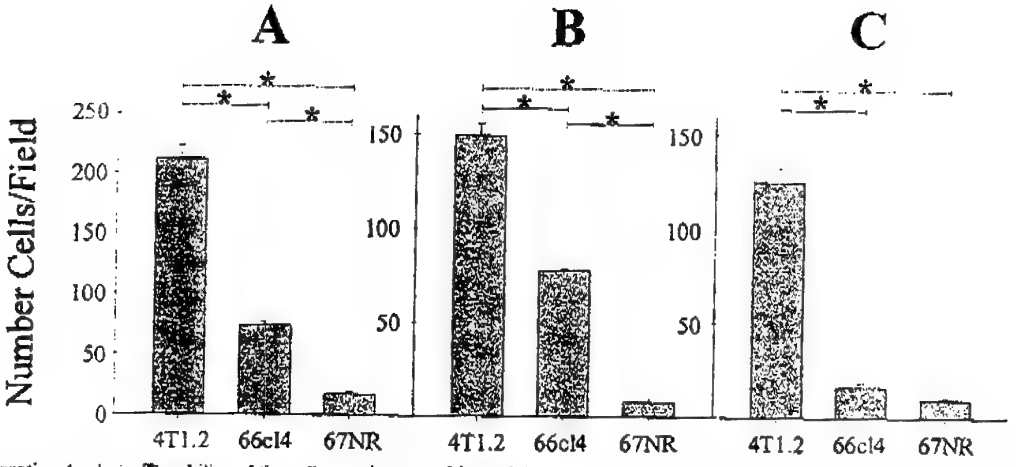


Figure 4. Migration Analysis. The ability of the cells to migrate on Matrigel (A), collagen type I (B) or IV (C) was analyzed by microchemotaxis. The assay was performed for 5 h. The bars represent the mean ±SEM ($n = 6$). Statistical analysis was performed by one way ANOVA and * indicates a P value <0.001.

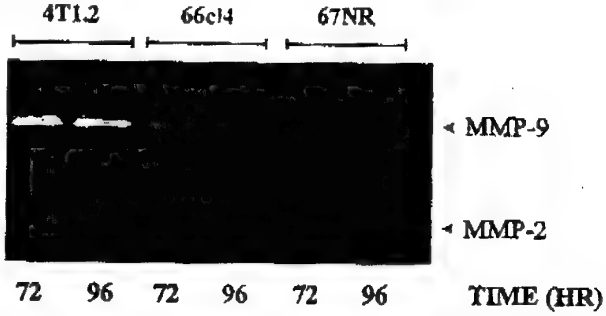


Figure 5. Gelatinase Production. Cells were cultured on plastic in SFM for 96 h. Conditioned media was collected and analyzed by gelatin zymography to detect any gelatinases (MMP-2 and 9) secreted by the cells.

was → were

breast cancer cell lines, and is certainly less polygonal than the non-invasive, epithelial like human breast cancer cell lines (e.g., MCF-7, T47D or MDA-MB-453 [22]). Thus, the morphological appearance of these cells and their expression of vimentin suggest that despite their lack of invasiveness and metastatic potential, their inability to invade may result either from a post-EMT modification or only partial onset of the EMT.

The *in vitro* migration and invasion analysis indicated that the 4T1.2 cells were significantly more motile on all the substrates analyzed, and were able to traverse a Matrigel coated filter more efficiently than the 66cl4 cells. The 67NR cell line was the least effective cell line at either migrating or invading a Matrigel barrier. Our previous studies have shown a correlation between stellate Matrigel outgrowth and invasiveness in established human breast cancer cell lines [22, 24] and in oncogene transformed mammary epithelial cells [25, 26]. The capacity of the 67NR cells for Matrigel outgrowth suggests that this is more closely related to their proliferative and morphological phenotype than their motility, and further supports the notion that these cells have many but not all the features of a metastatic cell. In addition, when 67NR cells are injected via intracardiac inoculation into mice, metastases are detected in the lung and occasionally in the spine of the mice (data not shown). This would suggest that the 67NR cells have the ability to establish and grow at a secondary site but are unable to disseminate from the primary tumour, perhaps because they lack the capability to migrate and invade basement membranes. In contrast to morphology and Matrigel outgrowth, motility and chemoinvasion analysis clearly distinguished the aggressively metastatic 4T1.2 cells from 66cl4 and 67NR.

The gelatinases MMP-2 and 9 are specifically able to degrade collagen type IV as well as a wide range of substrates, including collagen types V, VII and XI, fibronectin, laminin, elastin, proteoglycans and entactins to varying degrees [3, 27–29]. A strong association has been made between the production of gelatinases and the invasive and metastatic potential of a large variety of cancers including lung, prostate, breast, colon and neuroblastoma [30–34]. Analysis of the gelatinase production of the murine carcinoma cells *in vitro* showed that the more metastatic 4T1.2 line produced high levels of MMP-9 compared to the less metastatic 66cl4 and 67NR. While none of the cell lines produced detectable amounts of MMP-2, increased expression of MMP-9 has been reported in highly metastatic sublines of the 13762NF Fisher rat mammary adenocarcinoma [35]. MMP-9 may play an active role in metastasis in non mammary cancers as well [34, 36, 38], and detailed studies by Bernhard et al. [39] have clearly implicated MMP-9 in the metastatic capacity of ras-transformed Rat1 cells.

The gelatinases, like most of the MMPs, are synthesized in a latent pro-enzyme form, and associations have been made between the ability of cancer cells to activate the latent gelatinase and their metastatic/invasive phenotype [19, 40, 41]. Although a number of enzymes have been implicated in the activation of pro-MMP-9, including MMP-3, MMP-2, plasmin and tissue kallikrein [42-45], the mechanism is not well established. In contrast, the activation of MMP-2 has been well documented as a two step process. The first step involves cleavage of the latent 72 kDa MMP-2 at the cell surface by MT1-MMP to a 62 kDa intermediate form, which is subsequently autocatalytically converted to the fully mature

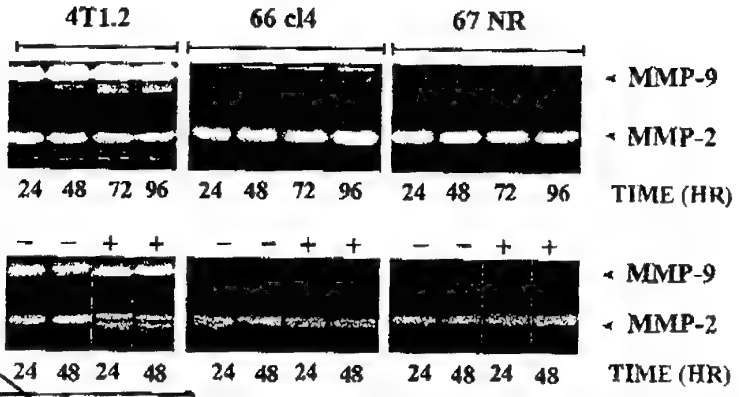


Figure 6. Time Course Analysis of Pro MMP-2 Activation. Cells were cultured either on a gel of fibrillar collagen (A) or on plastic in the presence (+) or absence (−) of Con A (B) in SFM supplemented with exogenous latent pro-MMP-2. Conditioned media harvested at various times was analyzed by gelatin zymography to determine if the cells were able to activate exogenous pro-MMP-2.

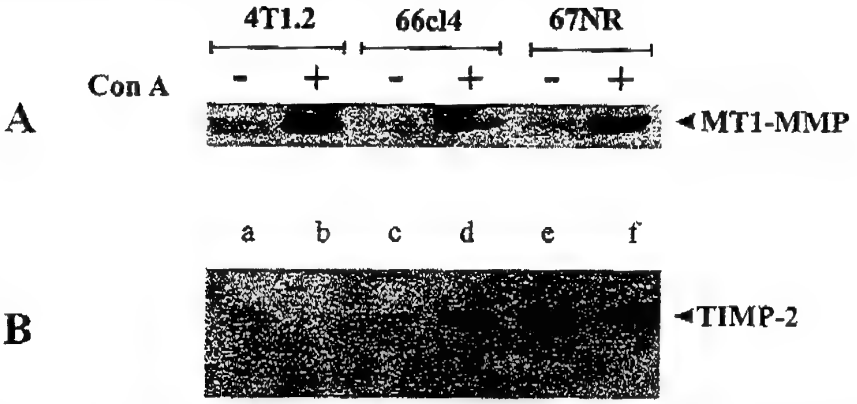


Figure 7. MT1-MMP and TIMP-2 Levels. (A) Whole cell lysates of the cells cultured on plastic in the presence (+) or absence (−) of Con A (20 μg/ml) were analyzed by Western blot using anti MT1-MMP antibody (114-1F2). (B) Cells were cultured in the presence of Con A (20 μg/ml), whole cell lysates (lanes a-c) and the conditioned media (lanes d–f) were analyzed by Western blot using anti TIMP-2 antibody (67-4H11). 4T1.2 cells are in lanes a and d, 66CL4 in lanes b and e and 67NR in lanes c and f.

59 kDa form [46, 47]. MMP-2 activation can be induced in cultured cells by a number of factors including Con A, phorbol ester, transforming growth factor β and collagen [47–50]. Analysis of the murine carcinoma cells showed a correlation between their ability to activate pro-MMP-2 and their *in vivo* metastatic potential, which is consistent with our observations in human breast cancer model systems [40]. However Western analysis indicated that all three cell lines produced relatively equal amounts of the MT1-MMP protein, and that Con A induced MT1-MMP protein levels equally in each cell line. Like the vimentin expression in 67NR, this is in contrast to human breast cancer cell lines when only invasive cells express MT1-MMP and activate MMP-2. Human breast cancer cell lines which cannot activate MMP-2 lack MT1-MMP. The lack of MMP-2 activation by the 67NR cells in response to either collagen or Con A suggests that the MT1-MMP, which is abundant in these cells, is either not functional or is restrained. The regulation of MMP-2 activation by MT1-MMP is complex and involves both transcriptional and non-transcriptional modulation of MT1-MMP [16, 19]. TIMP-2 is also an integral component of the activation complex [20, 51] and either an excess or relative lack of TIMP-2 may lead to inhibition of MMP-2 activation. We also observed that all three cell lines produced relatively equal quantities of TIMP-2. This would therefore suggest that the inability of the 67NR cells to activate MMP-2 is not due to an MT1-MMP/TIMP-2 imbalance and may relate to a lack of the $\alpha_v\beta_3$ integrin which has been shown to interact with MMP-2 [52] and may play a role in the activation of pro-MMP-2 [53]. Indeed, preliminary analyses have indicated that 4T1.2 cells express the $\alpha_v\beta_3$ integrin but the 66Cl4 and 67NR cells do not (Sloan and Anderson, unpublished data).

The *in vitro* behavior of these murine breast carcinoma cells in the Boyden chamber assays, as well as their gelatinase profiles, demonstrates a clear correlation with the *in vivo* metastasis data [12]. The 4T1.2 cell line that metastasizes to bone following orthotopic inoculation into the mammary gland exhibited the highest invasive potential, greatest motility, highest production of MMP-9, and the greatest ability to activate latent MMP-2 *in vitro* compared to the less metastatic 66cl4 and non-metastatic 67NR cell line. Since the 67NR cells exhibit many of the hallmarks of invasive and metastatic cells, their lack of MMP-9 and inability to activate MMP-2 may largely be responsible for their decreased motility, invasiveness and metastasis although the absence of additional factors cannot be ruled out. These

cell lines represent a valuable resource for further studies of these factors and further delineation of the EMT process.

Acknowledgements

This work was supported in part by the Victorian Breast Cancer Research Consortium, Inc., The Anti-Cancer Council of Victoria, The National Institutes of Health (R21-CA87244-01; EWT), and the United States Department of the Army (DAMD17-98-1-8144; RLA). The content of the information does not necessarily reflect the position or the policy of the US government and no official endorsement should be inferred.

References

1. Matrisian LM. Metalloproteinases and their inhibitors in tissue remodeling. Trends in Genet 1990; 6: 121–5.
2. Testa JE, Quigley JP. The role of urokinase-type plasminogen activator in aggressive tumour cell behavior. Cancer Metastasis Rev 1990; 9: 353–67.
3. Sloane BF, Moin K, Lah TT. Regulation of lysosomal endopeptidases in malignant neoplasia. In Pretlow TG and Pretlow TP (eds): Biochemical and Molecular Aspects of Selected Cancers. New York: Academic Press 1994; Vol. 2, pp. 411–66.
4. Terranova VP, Hujanen ES, Martin GR. Basement membrane and the invasive activity of metastatic tumour cells. J Natl Cancer Inst 1986; 77: 311–6.
5. Basset P, Bellocq JP, Wolf C et al. A novel metalloproteinase gene specifically expressed in stromal cells of breast carcinomas. Nature (London) 1990; 348: 699–704.
6. Pyke C, Ralfkiaer E, Tryggvason K et al. Messenger RNA for two type IV collagenases is located in stromal cells in human colon cancer. Am J Pathol 1993; 142: 359–65.
7. Heppner KJ, Matrisian LM, Jensen RA et al. Expression of most matrix metalloproteinase family members in breast cancer represents a tumor induced host response. Am J Pathol 1996; 149: 273–82.
8. Nielsen BS, Timshel S, Kjeldsen L et al. 92 kDa type IV collagenase (MMP-9) is expressed in neutrophils and macrophages but not in human colon cancer. Int J Cancer 1996; 65: 57–62.
9. Gilles C, Thompson EW. The epithelial to mesenchymal transition and metastatic progession in carcinomas. The Breast J 1996; 2: 83–96.
10. Yoneda T, Sasaki A, Mundy GR. Osteolytic bone metastasis in breast cancer. Breast Cancer Res 1994; 32: 73–84.
11. Miller BE, Roi LD, Howard LM, Miller FR. Quantitative selectivity of contact mediated intercellular communication in a metastatic mouse mammary tumor line. Cancer Res 1983; 43: 4102–7.
12. Lelekakis M, Moseley JM, Martin TJ et al. A novel orthotopic model of breast cancer metastasis to bone. Clin Exp Metastasis 1999; 17: 163–70.
13. Sung V, Cattell DA, Bueno JM et al. Human breast cancer cell metastasis to long bone and soft organs of nude mice: A quantitative assay. Clin Exp Metastasis 1997; 15: 173–82.
14. Kjonniksen I, Winderen M, Brunland O et al. Validity and usefulness of human tumor models established by intratibial cell inoculation in nude rats. Cancer Res 1994; 54: 1715–9.
15. Aslakson CJ, Miller FR. Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res 1992; 52: 1399–405.
16. Yu M, Sato H, Seiki M et al. Complex regulation of membrane-type matrix metalloproteinase expression and matrix metalloproteinase-2 activation by concanavalin A in MDA-MB-231 human breast cancer cells. Cancer Res 1995; 55: 3272–7
17. Sato H, Takino T, Okada Y et al. A matrix metalloproteinase expressed on the surface of invasive tumor cells. Nature (London) 1994; 370: 61–5.
18. Stetler-Stevenson WG. Type IV collagenases in tumor invasion and metastasis. Cancer Met Rev 1990; 9: 289–303.
19. Pulyaeva H, Bueno J, Polette M et al. MT1-MMP correlates with MMP-2 activation potential seen after epithelial to mesenchymal transition in human breast carcinoma cells. Clin Exp Metastasis 1997; 15: 111–20.
20. Sato H, Seiki M. Membrane-type-matrix metalloproteinase. J Biochem 1996; 119: 209–15.
21. Sommers CL, Thompson EW, Torri J et al. Absence of cell adhesion molecule uvomorulin expression in invasive human breast cancer cell lines. Cell Growth and Differentiation 1991; 2: 365–72.
22. Thompson EW, Paik S, Brunner N et al. Association of increased basement invasiveness with absence of estrogen receptor and expression of vimentin in human breast cancer cell lines. J Cell Physiol 1992; 150: 534–44.
23. Hay ED. An overview of epithelio-mesenchymal transformation. Acta Anat 1995; 154: 8–20.
24. Bae SN, Arand G, Azzam H et al. Molecular and cellular analysis of basement membrane invasion by human breast cancer cells in Matrigel-based *in vitro* assays. Breast Cancer Res Treat 1993; 24: 241–55.
25. Thompson EW, Torri J, Sabol M et al. Oncogene-induced basement membrane invasiveness in human mammary epithelial cells. Clin Exp Metast 1994; 12: 181–94.
26. Giunciuglio D, Culty M, Fassina G et al. Invasive phenotype of MCF-10A cells over expressing c-Ha-ras and c-erbB-2 oncogenes. Int J Cancer 1995; 63: 815–22.
27. Woessner JF. Matrix metalloproteinases and their inhibitors in connective tissue remodeling. FASEB J 1991; 5: 2145–54.
28. Murphy G, Cockett MF, Ward RV et al. Matrix metalloproteinase degradation of elastin, type IV collagen and proteoglycan. A quantitative comparison of the activities of 95 kDa and 72 kDa gelatinases, stromelysins -1 and -2 and punctuated metalloproteinase (PUMP). Biochem J 1991; 277: 277–9.
29. Sires UI, Griffin GL, Brockelman TJ et al. Degradation of entactin by matrix metalloproteinases: susceptibility to matrilysin and identification of cleavage sites. J Biol Chem 1993; 268: 2069–74.
30. Newell KJ, Witty JP, Rodgers WH et al. Expression and localization of matrix-degrading metalloproteinases during colorectal tumorigenesis. Mol Carcinog 1994; 10: 199–206.
31. Kawano N, Osawa H, Ito T et al. Expression of gelatinase A, tissue inhibitor of metalloproteinase-2, matrilysin and trypsin(ogen) in lung neoplasms: an immmunohistochemical study. Hum Pathol 1997; 28: 613–22.
32. Rha S, Kim JH, Roh JK et al. Sequential production and activation of matrix metalloproteinase -9 (MMP-9) with breast cancer progression. Breast Cancer Res Treat 1997; 43: 175–81.
33. Wood M, Fudge K, Mohler JL, et al. *In situ* hybridization studies of metalloproteinases 2 and 9 and TIMP-1 and TIMP-2 expression in human prostate cells. Clin Exp Metastasis 1997; 15: 246–58.
34. Sugiura Y, Shimada H, Seeger RC et al. Matrix metalloproteinases-2 and -9 are expressed in human neuroblastoma: contribution of stromal cells to their production and correlation with metastasis. Cancer Res 1998; 58: 2209–1.
35. Nakajima M, Welch DR, Wynn DM et al. Serum and plasma Mr 92,000 progelatinase levels correlate with spontaneous metastasis of rat 13762NF mammary adenocarcinoma. Cancer Res 1993; 53: 5802–7.
36. Zucker S, Lysik RM, Zarrabi MH et al. M_r 92,000 type collagenase is increased in plasma of patients with colon cancer and breast cancer. Cancer Res 1993; 53: 140–6.
37. Ueda Y, Imai K, Tsuchiya H et al. Matrix metalloproteinase 9 (gelatinase B) is expressed in multinucleated giant cells of human giant cell tumor of bone and is associated with vascular invasion. Am J Pathol 1996; 148: 611–22.
38. Zeng ZS, Huang Y, Cohen AM et al. Prediction of colorectal cancer relapse and survival via tissue RNA levels of matrix metalloproteinase-9. J Clin Oncol 1996; 14: 3133–40.
39. Bernhard EJ, Gruber SB, Muschel RJ. Direct evidence linking expression of matrix metalloproteinase 9 (92-kDa gelatinase/collagenase) to the metastatic phenotype in transformed rat embryo cells. Proc Natl Acad Sci USA. 1994; 91: 4293–7.
40. Azzam HS, Arand GA, Lippman ME et al. MMP-2 activation potential associates with metastatic progression in human breast cancer cell

lines, and is independent of MMP-2 production. J Natl Cancer Inst 1993; 85: 1758–64

41. Yamamoto M, Mohanam S, Sawaya R et al. Differential expression of membrane-type matrix metalloproteinase and its correlation with gelatinase A activation in human malignant brain tumors *in vivo* and *in vitro*. Cancer Res 1996; 56: 384–92.
42. Ogata Y, Enghild JJ, Nagase H. Matrix metalloproteinase 3 (stomelysin) activates the precursor for the human matrix metalloproteinase 9. J Biol Chem 1992; 267: 3581–4.
43. Fridman R, Toth M, Pena D et al. Activation of progelatinase B (MMP-9) by gelatinase A (MMP-2). Cancer Res 1995; 55: 2548–55.
44. Okada Y, Gonoji Y, Naka K et al. Matrix metalloproteinase 9 (92-kDa gelatinase/type IV collagenase) from HT1080 human fibrosarcoma cells. J Biol Chem 1992; 267: 21712–9.
45. Menashi S, Fridman R, Desrevieres S et al. Regulation of 92 kDa gelatinase B activity in the extracellular matrix by tissue kallikrein. Ann NY Acad Sci 1994; 732: 466–8.
46. Atkinson SJ, Crabbe T, Cowell S et al. Intermolecular autocatalytic cleavage can contribute to the activation of progelatinase A by cell membranes. J Biol Chem 1995; 270: 30479–85.
47. Overall CM, Sodek J. Concanavalin A produces a matrix-degradative phenotype in human fibroblasts. Induction and endogenous activation of collagenase, 72-kDa gelatinase, and PUMP-1 is accompanied by the tissue inhibitor of matrix metalloproteinases. J Biol Chem 1990; 265: 21141–51.
48. Strongin AY, Marmer BL, Grant GA et al. Plasma membrane dependent activation of the 72-kDa type IV collagenase is prevented by complex formation with TIMP-2. J Biol Chem 1993; 268: 14033–9.
49. Brown PD, Bloxide RE, Stuart NSA et al. Association between expression of activated 72-kilodalton gelatinase and tumor spread in non-small lung carcinoma. J Natl Cancer Inst 1993; 85: 574–8.
50. Azzam HS, Thompson EW. Collagen induced activation of the M_r 72,000 type IV collagenase in normal and malignant human fibroblastoid cells. Cancer Res 1992; 52: 4540–4.
51. Strongin AY, Collier I, Bannikov G et al. Mechanism of cell surface activation of 72-kDa type IV collagenase. Isolation of the activated form of the membrane metalloproteinase. J Biol Chem 1995; 270: 5331–8.
52. Brooks PC, Stromblad S, Sanders LC et al. Localization of matrix metalloproteinase MMP-2 to the surface of invasion cells by interaction with integrin $\alpha_v\beta_3$. Cell 1996; 85: 683–93.
53. Deryugina EI, Bourdon MA, Jungwirth K et al. Functional activation of integrin alpha V beta 3 in tumor cells expressing membrane-type 1 matrix metalloproteinase. Int J Cancer 2000; 86(1): 15–23.

Parathyroid hormone related protein (PTHrP) expression does not dictate the ability of breast cancer cell lines to metastasise to bone.

Michael D. Tavaria[1], Maria Lelekakis[1], Anthony Natoli[1], Erica Sloan[1], Patricia Ho[2], Daphne Hards[2], T. John Martin[2], Jane M. Moseley[2] and Robin L. Anderson[1]

[1]Trescowthick Research Laboratories, Peter MacCallum Cancer Institute, Melbourne, Victoria, 8006, Australia

[2] St Vincent's Institute of Medical Research, Fitzroy, Victoria, 3065, Australia

INTRODUCTION

Despite improved diagnostic imaging technologies, metastatic disease remains a common and serious complication for those diagnosed with malignant primary breast carcinoma. Breast cancer metastasis involves several sites including lymph nodes, lung, liver and particularly bone. Bone is the most common distal site of breast cancer metastasis and osteolytic bone metastases are present in around two-thirds of patients with late stage disease. It is over a century ago since Paget noted the proclivity of breast cancer for destructive bone metastasis, yet the mechanisms underlying this phenomenon are yet to be fully understood. One of the factors impeding progress in this regard has been the lack of suitable animal models that mimic the entire metastatic process.

We have reported the initial characterisation of an orthotopic mouse model of breast cancer metastasis to bone (Lelekakis et al., 1999) in which tumour cells are implanted into the mammary fat pad of syngeneic mice. This model is unique in that the primary breast tumours spontaneously metastasize to secondary sites resembling those in human patients. Of particular interest is the observation that bone-resorbing osteoclasts are recruited to bone surfaces adjacent to metastatic tumour deposits resulting in osteolysis and hypercalcemia, also characteristic of human metastatic breast cancer. This model provides a powerful tool for investigating mechanisms of breast cancer metastasis to bone and for testing novel anti-metastatic agents.

One of the factors commonly associated with the ability of breast cancer cells to metastasize to bone is parathyroid hormone related protein (PTHrP). PTHrP was first identified as the factor mediating humoral hypercalcemia of malignancy (HHM) (Henderson et al, 1989, Suva et al, 1987). PTHrP actually comprises several isoforms created by alternative splicing (Moseley et al, 1987), all of which contain an NH_2 terminal region homologous to that of

PTH, a major regulator of calcium homeostasis (Jueppner et al, 1991, Abou-Samra et al, 1992). It is through this region that both molecules bind a common G-protein-coupled seven transmembrane PTH/PTHrP receptor and mediate similar biological effects. Unlike PTH that is produced almost exclusively by the parathyroid gland, PTHrP is widely expressed in normal foetal and adult tissues, where it has been implicated in paracrine regulation of growth and differentiation (Jueppner et al, 1991, Abou-Samra et al, 1992, Burton et al, 1990, Kaiser et al, 1994). The PTH/PTHrP receptor is widely expressed, but most abundant in renal tubule cells, chondrocytes and osteoblasts where it functions to regulate calcium homeostasis and bone remodelling.

Bone remodelling is regulated through the expression of a group of recently identified molecules including the osteoclast differentiation factor, receptor activator of NF-κB ligand (RANKL), and osteoprotegerin (OPG) by osteoblasts (Lacey et al., 1998, Yasuda et al., 1998, Hsu et al., 1999). RANKL is a membrane-bound TNF-related molecule expressed on the cell surface of osteoblasts that binds the receptor, RANK, that is present on osteoclast precursor cells and stimulates osteoclast differentiation and survival. RANKL also binds the soluble decoy receptor, OPG, a secreted product of osteoblasts (Tsuda et al., 1997, Simonet et al., 1997). Thus, osteoblasts regulate osteoclastogenesis by balancing the production of RANKL and OPG. Factors that stimulate production of RANKL and therefore favour osteoclastogenesis include interleukins (IL) 1, 6 and 11, prostaglandin E2 and PTHrP.

Studies using well established mouse models of breast cancer growth in bone and observations of human osteolytic bone metastases suggest that the osteolysis associated with bone metastases is mediated through the osteoblast. Indeed, overexpression of PTHrP expression in breast cancer cells has been shown to enhance osteolysis (Guise et al, 1996, Thomas et al, 1999) and stimulate osteoclastogenesis via increased osteoblast expression of RANKL (Quinn et al, 2000, Martin and Gillespie, 2001). Osteolysis also releases TGFβ stored in bone, leading to stimulation of tumour cell growth and production of PTHrP, which in turn further increases osteoblastic production of RANKL and consequently stimulates more osteoclastogenesis (Yin et al., 1999). These studies therefore implicate PTHrP, TGFβ and RANKL as important mediators of breast cancer mediated osteolysis. PTHrP expression has been reported in approximately two thirds of primary breast cancers, leading to the suggestion that PTHrP may be an important prognostic indicator for bone

metastases (Bundred et al, 1992, Guise et al, 1996). However, recent clinical data (Henderson et al, 2001) suggesting that breast tumour PTHrP expression correlates with improved prognosis has focussed attention on the importance of local microenvironment factors on expression of critical determinants of metastatic growth.

This study investigates the role of PTHrP in a unique orthotopic mouse model of breast cancer metastasis to bone. Using a variety of tumour cell implantation routes, genetic manipulation of PTHrP expression levels in tumour cell lines and a quantitative assay for metastatic tumour burden, we demonstrate in this model that PTHrP expression is not a determinant of whether a tumour cell is able to metastasize to bone. These data have important implications for the use of PTHrP as a marker of clinical disease and as an anti-metastasis agent in cases of breast cancer metastasis to bone.

MATERIALS and METHODS

Cell Culture

Four tumour cell lines are described in this study. Essentially, 67NR is non-metastatic, 66cl4 metastasizes to lymph nodes and lungs, 4T1.2 and 4T1.13 metastasize to lymph nodes, lung, liver and bone. The characterisation of these tumour cell lines has been previously described (Lelekakis *et al*, 1999) with the exception of 4T1.13, which is a subclone of 4T1 derived by single cell cloning with similar metastatic properties to 4T1.2. Tumour cell lines were cultured in αMEM medium supplemented with 10% foetal calf serum and maintained in incubators at 37°C and 5% CO_2. Cells were not allowed to reach confluence and were passaged for a maximum of 4 weeks to minimise genetic drift.

Histology

Primary tumour, lung, spine, tibia and femur samples were fixed in 10% buffered formalin. Bones were decalcified in EDTA (Kartsogiannis et al., 1997) and embedded in paraffin. Standard haematoxylin and eosin (H&E) staining procedures were used to assess morphology.

Tumour Cell Inoculation and Growth

A. Intramammary fat pad (imfp) injections were used to investigate metastasis after orthotopic implantation of tumour cells. Six-eight week old female Balb/c mice were briefly anaesthetized and $1x10^5$ tumour cells injected into the fourth mammary fat pad in a

20µl volume containing 10µl of matrigel. Tumours were allowed to develop for periods of 25 – 38 days depending on the tumour cell line.

B. Intratibial (it) injections were used to investigate the ability of tumour cells to grow in bone. Four to five week old female Balb/c mice were anaesthetized and $5x10^3$ cells injected directly into the tibial shaft in a 20 µl volume. Tumour cells were allowed to grow for 14 – 18 days.

Quantitative Analysis of Metastatic Tumour Burden

A. Genomic DNA preparation.

Frozen tissues were first pulverized in steel homogenizers to produce a fine powder. Approximately 50mg aliquots of the ground tissue samples were then digested overnight with proteinase K in a NaCl/SDS buffer at 55°C. DNA was extracted from organs using a saturated salt precipitation method. DNA was diluted to approximately 100pg/µl in 2.5mMTris-HCl/0.25mM for analysis.

B. Real-time quantitative PCR (RTQ-PCR).

This assay is based on the ability of RTQ-PCR to measure the number of copies of the neomycin resistance gene (neo^r), present only in the transfected tumour cells, relative to that of an endogenous gene that is present in all cells. Primers and fluorescent probes to neo^r and vimentin were designed with the PrimerExpress software to utilise the TaqMan 5' fluorogenic assay method. Testing, optimisation and assays were all performed on a Perkin Elmer ABI Prism7700 (provided by Howard Florey Institute for Physiology and Medicine, Melbourne).

C. Relative tumour burden calculations.

Relative tumour burden (RTB) calculations were based on the comparative Ct method using a multiplex reaction. For each sample, the threshold cycle (C_t) value of the neo^r amplification profile was subtracted from the C_t value of the vimentin amplification profile to provide the ΔC_t value. The ΔC_t value represents the difference in the number of PCR cycles required for the two amplicons to reach a specified threshold. A corrected ΔC_t value ($Corr\Delta C_t$) for each group was then calculated to account for the different copy number of neo^r in each cell line. Since each cycle represents a twofold difference in the abundance of the target sequence, the relative tumour burden could then be calculated using the following formula:

$$RTB = 1/2^{Corr\Delta Ct} \times 10000$$

A multiplication factor of 10,000 was applied to this equation to produce a final RTB value that generally falls in the range of 0-10000. The RTB for each treatment group was then calculated by taking the average of individual RTB values. Standard errors of the mean (SEM) and p-values were calculated using the standard T-test.

PTHrP Expression Constructs and Assays

A. PTHrP Expression Constructs

A cDNA spanning the region coding for amino acids –36 to 139 of the human PTHrP protein cloned into the HindIII site of the mammalian expression vector pcDNA1neo (provided by M. Gillespie, SVIMR) was used to overexpress PTHrP in tumour cell lines. An antisense construct with the cDNA cloned in the reverse orientation was used to suppress endogenous PTHrP expression and pcDNA1neo was used as a vector control. Plasmids were introduced into the tumour cell lines by transfection with the non-liposomal FuGene6 reagent (Roche Diagnostics, Australia). Transfected cells were selected in media containing 300μg/ml G418 and single cell cloning of stable transfectants was achieved by dilution plating.

B. PTHrP Radioimmunoassay

PTHrP was measured in blood plasma or conditioned (serum free) tissue culture medium of cells in exponential phase using an N-terminal directed antibody radioimmunoassay as previously described (Grill et al, 1992). PTHrP levels in conditioned media were normalized to total cell protein by lysis of cells in 0.4M NaOH followed by a Lowry protein assay.

C. PTHrP Immunostaining

PTHrP immunostaining was performed as previously described (MacIsaac et al, 1991) using a sheep polyclonal antibody raised against synthetic human PTHrP (amino acids 50-69) at dilutions of 1/200 and 1/300 overnight at 4°C. Normal sheep serum was used for non-immune controls.

Plasma Calcium Measurement

Total plasma calcium levels were measured using an Arsenazo III assay (Trace Scientific, Australia).

RESULTS

PTHrP Expression and Metastatic Potential of Tumour Cell Lines.

We have previously shown a correlation between PTHrP secretion levels in the conditioned medium of cultured tumour cells and metastasis to bone of the tumour cell lines 67NR, 66cl4 and 4T1.2 (Lelekakis et al, 1999). Single cell cloning was used to produce 4T1.2 and several other 4T1 derived tumour lines. To investigate the correlation between PTHrP expression and metastasis, PTHrP secretion was measured in conditioned medium of several of these subclones. PTHrP secretion levels of these subclones ranged from 10 pmol/l/mg of protein (similar to 4T1.2) to baseline levels (<2pmol/l/mg) as in the case of 4T1.13 (Figure 1a). Interestingly, all 4T1 subclones tested demonstrated the ability to metastasize to bone approximately 28 days after imfp injection, regardless of *in vitro* PTHrP secretion levels (data not shown). Thus, *in vitro* PTHrP secretion measurement proved a poor indicator of bone metastasis, suggesting that PTHrP expression may be regulated *in vivo* by the local microenvironment. Indeed, the primary tumours of tumour lines that do not metastasize to bone and that secrete low levels of PTHrP *in vitro*, such as 66cl4 and 67NR, show similar degrees of PTHrP immunostaining to those of the bone metastasising tumour lines 4T1.2 and 4T1.13 (Figure 1b). Metastatic tumour cell deposits in bone also exhibit strong PTHrP immunostaining (Figure 1c) suggesting upregulation of PTHrP in the bone microenvironment.

To measure the effects of manipulating PTHrP expression on metastatic potential of the tumour lines, we developed a real time quantitative PCR (RTQ-PCR) assay. In this assay, genomic DNA is extracted from tissues of interest and real time PCR is used to determine the relative number of tumour cells present in a sample population. Comparison of this technique with established (non-quantitative) methods of detecting metastatic tumour burden such as histology showed good concordance, providing strong evidence of the reliability of using this method to measure metastatic tumour burden.

Effects of Altering PTHrP Expression on Metastasis of 4T1.13 to Bone.

To investigate the *in vivo* requirement for PTHrP expression, the tumour cell line 4T1.13 was transfected with PTHrP sense and antisense expression constructs. Single cell clones were isolated and i*n vitro* PTHrP secretion levels measured. One vector control (4T1.13c2), one overexpression (4T1.13s2) and two antisense (4T1.13as2 and 4T1.13as5) clones were selected for *in vivo* testing in the orthotopic mouse model of metastasis. *In vitro* secretion levels for these clones are shown in Figure2a. PTHrP expression was undetectable for the vector only

control and the two antisense expressing clones and in 4T1.13s2 was elevated to 15 pmol/l/mg of protein.

The ability of altered PTHrP expression to affect metastasis to bone was investigated by implanting the 4T1.13con2, 4T1.13sen2, 4T1.13as2 and 4T1.13as5 subclones into the mammary fat pad of female Balb/c mice. Four weeks after implantation, ten mice from each group were culled. Primary tumours were weighed, blood samples were taken for plasma PTHrP and calcium measurements and spines harvested for PCR analysis (Table 1, Figure 2b). The average primary tumour weight in mice injected with 4T1.13as2 and 4T1.13as5 was slightly but significantly ($p<0.05$) less than mice injected with 4T1.13con2. No significant differences were observed in the parameters of plasma PTHrP or plasma calcium levels between any of the groups. Real-time quantitative PCR (RTQ-PCR) measurement revealed a significant ($p<0.001$) reduction in relative tumour burden (RTB) in the spines of mice implanted with 4T1.13 cells expressing PTHrP antisense DNA (4T1.13as2 and 4T1.13as5) compared to controls (4T1.13con2). No difference in RTB was observed between mice injected with 4T1.13 cells overexpressing PTHrP and controls (4T1.13sen2 versus 4T1.13con2).

To determine if the reduced size of primary tumours in mice injected with 4T1.13as2 and 4T1.13as5 was caused by altered growth rates of the cells, *in vitro* growth assays were performed (Figure 2c). Equal numbers of cells were seeded into 96 well plates and allowed to grow for four days. Cell density was measured at 24 hour intervals using a sulpharhodamine B (SRB) colourimetric assay. Using this method, the *in vitro* growth rate of 4T1.13sen2 was identical to 4T1.13con2. However, the growth rates of both tumour lines expressing antisense PTHrP were significantly reduced ($p<0.001$). These data suggest that the reduced tumour size and hence bone metastatic tumour burden of mice injected with 4T1.13as2 and 4T1.13as5 may be due to an effect of PTHrP inhibition on growth rate of the tumour cells.

To further investigate the effects of manipulating PTHrP expression on the growth of 4T1.13 tumour cells, cells were injected directly into the tibia of 4 to 5 week old female Balb/c mice. Eighteen days later, tibia were removed and growth of the tumour cells was measured using RTQ-PCR (Figure 2d). No significant differences were observed in the growth of tumour cells between control and either PTHrP sense or antisense transfected groups.

Effects of Altering PTHrP Expression on Metastasis of 66cl4 to Bone.

The tumour cell line 66cl4 metastasizes to lymph nodes and lung, but does not produce overt bone metastases. To determine whether the failure to successfully colonise bone was due to an inability to upregulate PTHrP expression, subclones were generated by transfection of 66cl4 with a PTHrP overexpression construct. Several subclones secreting markedly increased *in vitro* PTHrP levels compared to base vector transfected 66cl4 were isolated (Figure 3a). *In vitro* growth rates of PTHrP overexpressing tumour lines were not significantly different to parental 66cl4 (Figure 3b).

These subclones were injected into the mammary fat pad of female Balb/c mice and tumours allowed to develop for 32 or 39 days. The experimental groups culled at day 32 were 66cl4sen40 and 66cl4sen100. These mice were euthanized due to deterioration in condition. The remaining groups were euthanized 7 days later. Fifteen mice from each group were culled. Primary tumours were weighed, blood samples were taken for plasma PTHrP and calcium measurements and spines harvested for PCR analysis (Figure 3b). No significant difference between control and transfected groups was observed for the parameter of primary tumour weight, except for 66cl4sen40 and 66cl4sen100, which were euthanized 7 days prior to the remaining groups. Primary tumour size was proportionally reduced in these groups. Despite this difference in primary tumour weight, mice injected with these tumour lines displayed increased plasma PTHrP and Ca^{++} levels compared to mice injected with vector only transfected controls (Table 2). One other group, 66cl4sen10, displayed elevated plasma PTHrP but not plasma Ca^{++} levels.

Metastatic tumour burden in the spines of mice injected with 66cl4 subclones overexpressing PTHrP was compared to that of mice injected with vector only transfected 66cl4 using RTQ-PCR (Figure 3c). Consistent with previous observations that 66cl4 metastasizes poorly to bone, low levels of tumour burden were detected in mice injected with the control lines and three PTHrP overexpressing lines. One PTHrP overexpressing line (66cl40s100) produced elevated tumour burden in mice, however this was not statistically significant ($p=0.14$). These data suggest that overexpression of PTHrP in 66cl4 tumour cells is insufficient to alter the metastatic potential of this tumour cell line with respect to bone.

To determine if the failure of 66cl4 and its PTHrP overexpressing derivatives to metastasise to bone in our model was due to an inability to proliferate in the bone microenvironment,

these tumour lines were injected directly into the tibia of 6 week old mice. Eighteen days later, the tibia were removed and tumour burden measured by RTQ-PCR (Figure 3d). The relative tumour burden present in both control and PTHrP overexpressing groups was considerably higher than when intramammary fat pad was used as the route of injection, suggesting that 66cl4 cells proliferate in the bone microenvironment. However, there was no difference in relative tumour burden in the tibia of the control group compared to PTHrP overexpressing groups, suggesting that PTHrP does not modulate this proliferation.

DISCUSSION

The properties of tumour cells that promote metastasis to specific secondary sites remain largely unknown. Identification of the factors responsible for this tropism has proven particularly difficult in the case of breast cancer cells that commonly metastasise to bone. Bone is a complex microenvironment undergoing constant remodelling. This remodelling is influenced by a plethora of locally and systemically acting cytokines and hormones that regulate the expression of effector molecules such as RANKL, OPG and RANK by osteoblasts and osteoclasts. In particular, the differentiation, survival and activity of osteoclasts are tightly regulated through these molecules. Current dogma and recent experimental evidence suggest that metastatic tumour cells disturb this delicate balance by producing factors that stimulate osteoclastogenesis. One of the factors produced by many breast cancer cells and implicated in this process is PTHrP (Thomas et al., 1999, Yin et al., 1999).

We have developed a unique and clinically relevant animal model of breast cancer metastasis to bone (Lelekakis et al., 1999) that provides a novel system in which to investigate the importance of individual genes on this process. The tumour lines that comprise our mouse model of breast cancer metastasis are derived from the same spontaneous mammary carcinoma in a Balb/c mouse and are therefore expected to be genetically similar. Their different patterns of metastasis, however, point to the existence of differences in expression of genes that are important in the metastatic process. The ability to study gene function in this model system relies heavily on the availability of a method for quantitative evaluation of the effects of altering gene expression on the metastatic potential of the tumour cells. To this end, we developed a real time quantitative PCR assay based on amplification of the neomycin resistance gene that is present only in transfected tumour cells and an endogenous gene (vimentin) that is present in all cells. Comparison of the relative tumour burden using this

technique with other (non-quantitative) methods, such as histology, were performed and showed good concordance. The study described herein makes use of this method to quantify the effects of altered PTHrP expression on metastasis to bone in a unique mouse model.

PTHrP expression has been postulated to confer on breast tumour cells the ability to metastasise to bone. Indeed, PTHrP expression in a primary breast cancer was thought to be a poor prognostic marker indicating increased likelihood of bone metastases. However, recent data from a large clinical study suggests that this is not the case (Henderson et al., 2001). Instead, it appears that regulation of PTHrP expression in the bone microenvironment may be crucial to tumour colonisation of the bone. Interestingly, and in agreement with the aforementioned study, we show in our mouse model that primary tumours that do not metastasise to bone express PTHrP in apparently similar quantities to bone metastasising tumours and that high expression by a primary tumour is not predictive of bone metastasis.

Failure to attach to the appropriate vascular endothelium, to extravasate, invade or degrade bone or to proliferate may explain the inability of some tumour lines to colonise bone. Since PTHrP has been implicated in breast tumour cell growth and the ability of tumour cells to activate bone resorption, we investigated whether bone metastasis by the tumour line 4T1.13 was dependent on PTHrP. Transfection of an antisense PTHrP construct into 4T1.13 caused significant inhibition of growth *in vitro*. This reduced growth rate was reflected in slightly, but significantly, smaller primary tumour masses. However, *in vivo* growth of the antisense expressing tumour lines was unchanged as measured by tumour burden after intratibial injection. These data reveal the different responses of tumour lines to *in vitro* and *in vivo* growth conditions and emphasize the importance of local microenvironment. Bone metastases were markedly reduced in PTHrP antisense transfected tumour lines after imfp injection. This data implies that

Large tumours require angiogenesis to provide blood supply and prevent central necrosis. This process also provides additional escape routes for metastatic tumour calls. Therefore, size may be an important parameter in determining the metastatic potential of a primary tumour. Indeed, primary tumour size is a parameter used to determine clinical stage and treatment options for breast cancer patients. Thus, it is possible that reduced tumour growth could be responsible for delayed angiogenesis and therefore for reduced metastasis.

To determine whether PTHrP could influence the ability of a non-bone metastasizing tumour line to metastasise to bone, PTHrP was overexpressed in the tumour line 66cl4. This cell line normally metastasises to lymph nodes and lung, but not bone. Overexpression of PTHrP had no effect on bone metastasis of several such subclones of this cell line, despite elevated circulating PTHrP and hypercalcemia. Thus, PTHrP overexpression appears insufficient to direct tumour cells to metastasise to bone.

The deficiency in the 66cl4 cells responsible for their inability to metastasize to bone is likely to be in one of the later stages of metastasis such as adhesion or proliferation in the bone microenvironment since these cells metastasise successfully to lymph and lung. By injecting the cells directly into the tibia, we have shown that these tumour cells are capable of proliferation in the bone microenvironment. Indeed, these cells grow as well, if not better, in this environment than cells that metastasize to bone such as 4T1.13. These data imply that 66cl4 tumours fail to metastasise to bone because of a failure to properly access the bone microenvironment. Possible candidate gene families for this property include cell adhesion molecules such as the integrins and/or matrix metalloproteases such as MMP2 and MMP9. The role of these molecules in metastasis to bone in our mouse model is currently under investigation.

***Figure 1a. In vitro* PTHrP expression by tumour cell lines.** Secretion of PTHrP into culture medium of exponentially growing cells was measured by radioimmunoassay and normalized to total protein content of the sample.

***Figure 1b. In vivo* PTHrP expression in primary tumours.** Expression of PTHrP in primary tumours of different metastatic properties was detected by immunostaining with a peroxidase conjugated anti-synthetic human PTHrP antibody. Cell sections were stained with haematoxylin and eosin. All sections were photographed at 80x magnification. **A.** 67NR. **B.**66cl4. **C.** 4T1.2. **D.** 4T1.13

***Figure 1c.* PTHrP expression in bone metastases.** PTHrP immunostaining of sections through spine of a mouse bearing a 4T1.13 primary tumour. Left: section showing normal bone and bone marrow. Right: adjacent section showing large tumour deposit expressing PTHrP (brown staining).

***Figure 2a. In vitro* PTHrP expression by transfected tumour cells.** PTHrP was measured by radioimmunoassay in the culture medium of exponentially growing single cell clones of 4T1.13 transfected with vector only (con2), or PTHrP antisense (as2 and as5) and sense (sen2) expression constructs. PTHrP levels were normalized to total protein content.

***Figure 2b.* Bone metastases in mice bearing 4T1.13 primary tumours.** RTQ-PCR analysis of spines from mice injected with the transfected 4T1.13 clones described in Figure 2a.

***Figure 2c. In vitro* growth curves of transfected 4T1.13 clones.** Growth of parental 4T1.13 and the transfected 4T1.13 clones was measured using the SRB colourimetric assay. A student t-test applied to the day 4 results showed significant ($p<0.001$) difference between both 4T1.13as2 and 4T1.13as5 and 4T1.13con2

***Figure 2d. In vivo* growth of transfected 4T1.13 clones.** Transfected 4T1.13 tumour cells were injected directly into the tibia of 5 week old mice. Sixteen days later, tibia were removed and growth of the tumour cells was analysed by RTQ-PCR.

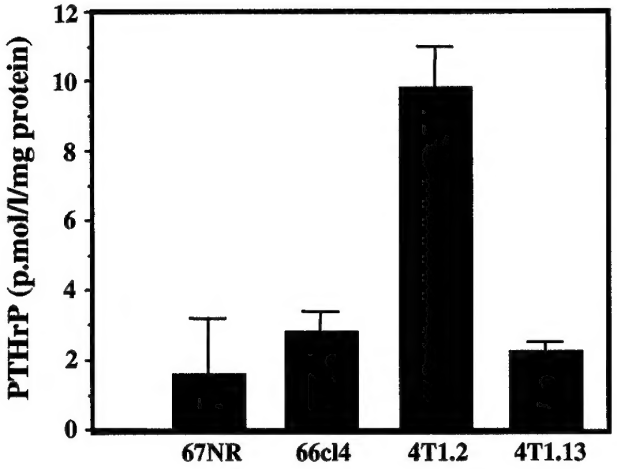
***Figure 3a. In vitro* PTHrP expression by transfected 66cl4 tumour cells.** PTHrP was measured by radioimmunoassay in the culture medium of exponentially growing single cell clones of 66cl4 transfected with vector only (con20 and con40), or PTHrP sense (sen10, sen40, sen100) expression constructs. PTHrP levels were normalized to total protein content.

***Figure 3b. In vitro* growth curves of transfected 66cl4 clones.** Growth of parental 66cl4 and the transfected 66cl4 clones was measured using the SRB colourimetric assay. No significant difference was observed.

***Figure 3c*. Bone metastases in mice bearing 66cl4 primary tumours.** RTQ-PCR analysis of spines from mice injected with the transfected 66cl4 clones described in Figure 3a.

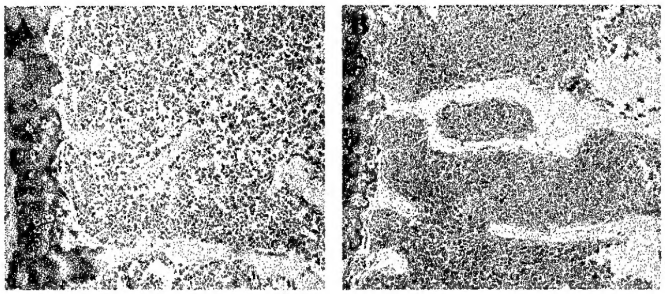
***Figure 3d. In vivo* growth of transfected 66cl4 clones.** Transfected 66cl4 tumour cells were injected directly into the tibia of 5 week old mice. Sixteen days later, tibia were removed and growth of the tumour cells was analysed by RTQ-PCR.

Figure 1



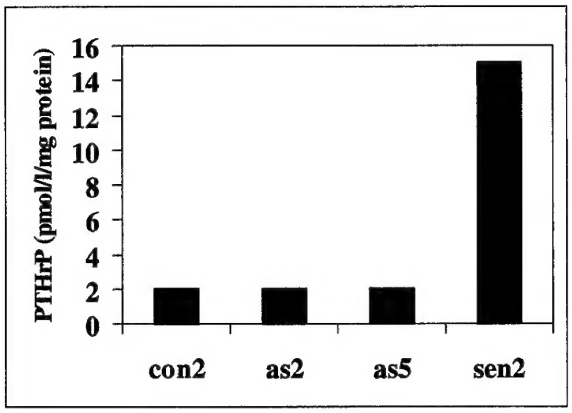
A

B

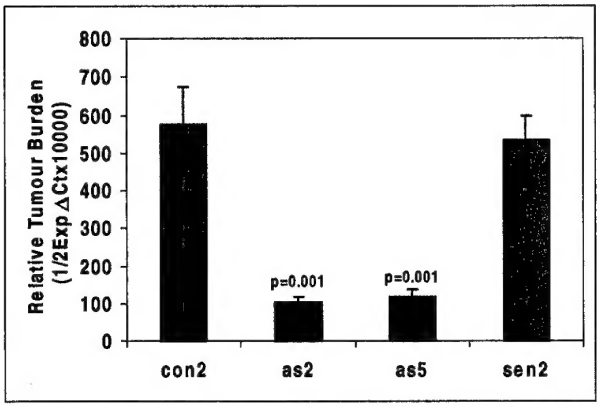


C

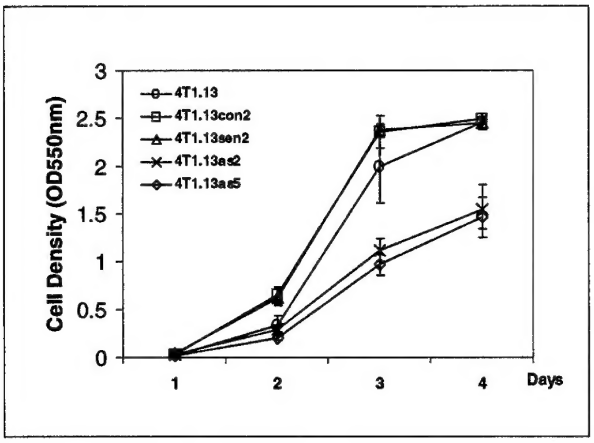
Figure 2



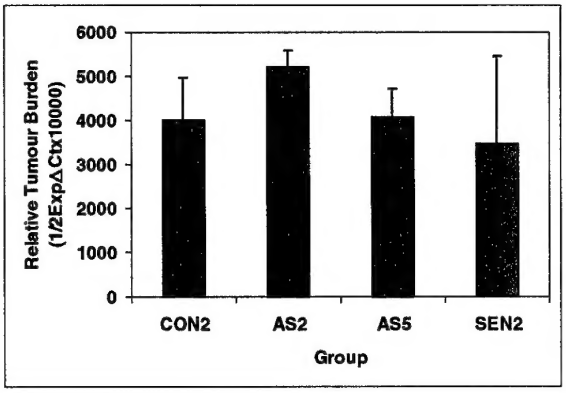
A



B

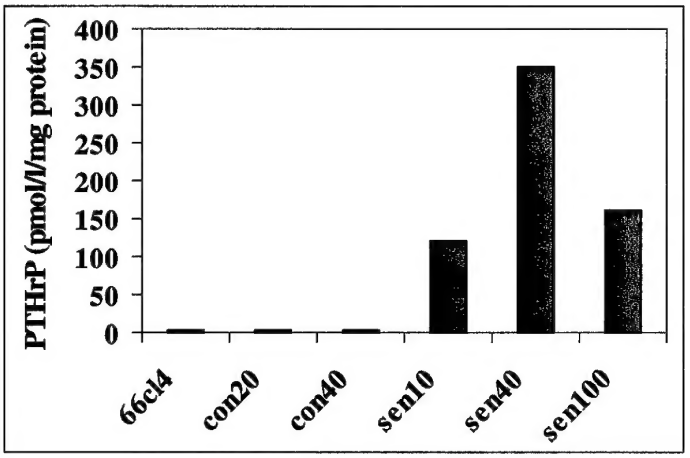


C

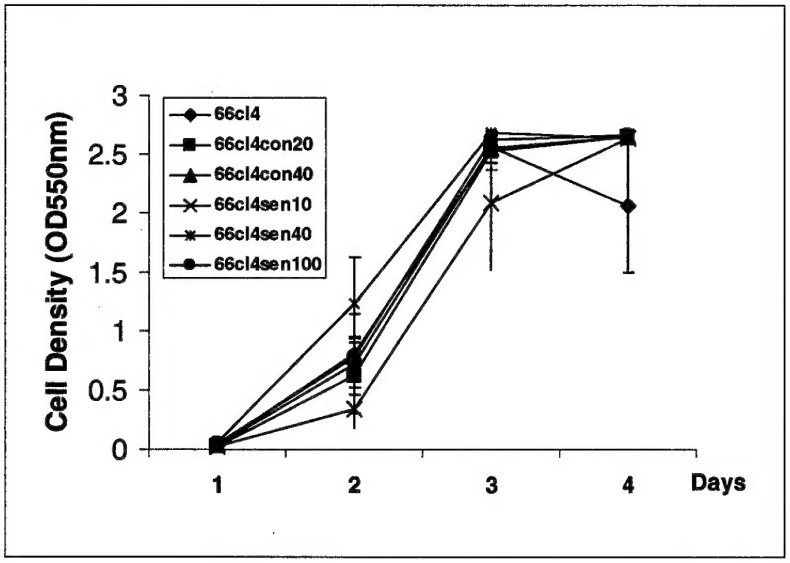


D

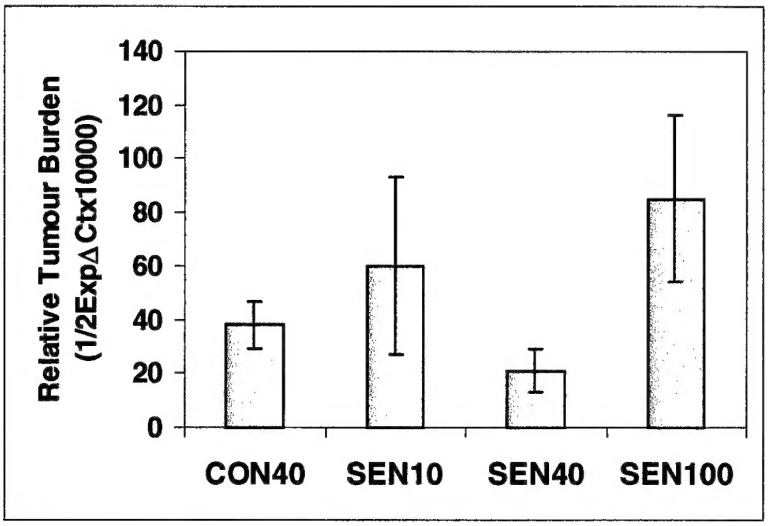
Figure 3


PTHrP (pmol/l/mg protein)
400
350
300
250
200
150
100
50
0
66cl4
con20
con40
sen10
sen40
sen100

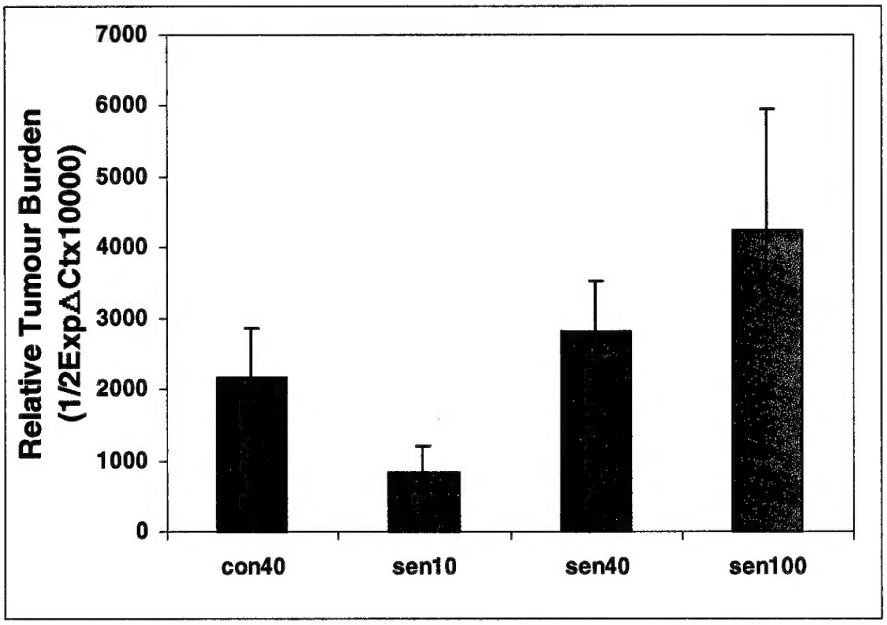
A


Cell Density (OD550nm)
3
2.5
2
1.5
1
0.5
0
66cl4
66cl4con20
66cl4con40
66cl4sen10
66cl4sen40
66cl4sen100
1
2
3
4
Days

B


Relative Tumour Burden
(1/2ExpΔCtx10000)
140
120
100
80
60
40
20
0
CON40
SEN10
SEN40
SEN100

C


Relative Tumour Burden
(1/2ExpΔCtx10000)
7000
6000
5000
4000
3000
2000
1000
0
con40
sen10
sen40
sen100

D

Fig. 4